

Melanocortin 1 receptor selective compounds

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The present invention relates to new compounds having high affinity and selectivity for the melanocortin 1 receptor (MC1-receptor). The new compounds selectively activate or block the MC1-receptors, which makes it possible to discriminate between the physiological effects mediated by the MC1-receptors and other subtypes of melanocortin receptors, and to afford selective pharmacological actions upon their administration to an animal or a human. The invention also relates to methods for their manufacture and their pharmaceutical preparations, as well as to their use for the treatment of medical and veterinary conditions which can be influenced by the MC1-receptor.

Related Art

Melanocortic peptides (melanocortins) are natural peptide hormones of animals, in particular mammals including man, which bind to and stimulate MC-receptors. Examples of melanocortins are  $\alpha$ -MSH,  $\beta$ -MSH,  $\gamma$ -MSH, ACTH, and their peptide fragments.  $\alpha$ -MSH is mainly known for its ability to regulate peripheral pigmentation (Eberle 1988), whereas ACTH is known to induce steroidogenesis (Simpson and Waterman 1988). The melanocortic peptides also mediate a number of other physiological conditions. Thus, they are reported to act as immunomodulators and to affect one or more of motivation, learning, memory, behaviour, inflammation, body temperature, pain, perception, blood pressure, heart rate, vascular tone, brain blood flow, nerve growth, placental development, aldosterone synthesis and release, thyroxine release, spermatogenesis, ovarian weight, prolactin and FSH secretion, uterine bleeding in women, sebum and pheromone secretion, blood glucose levels, weight homeostasis, intrauterine fetal growth as well as other events surrounding parturition (Garrud et al., 1974, Wiegant et al., 1979, O'Donahue et al., 1981, O'Donahue & Dorsa 1982, DeWied & Jolles 1982, Klein et al., 1985, Feng et al. 1987, Lin et al. 1987, Eberle, 1988, Gruber & Callahan 1989, DeWildt et al., 1995, Friedman 1997).

By the use of molecular cloning, genes encoding five different subtypes of MC-receptors have been identified (Chhajlani et al. 1993, Chhajlani and Wikberg 1992, Gantz et al. 1993a, b, Mountjoy et al. 1992). The MC-receptors belong to the class of G-protein coupled receptors which are all built from a single peptide forming 7 transmembrane domains. The five MC-receptors are termed MC1, MC2, MC3, MC4 and MC5 and they all couple in a stimulatory fashion to cAMP. Of these the MC2-receptor is the ACTH-receptor whereas the others constitute subtypes of melanocyte stimulating hormone receptors (MSH-receptors).

5 The MC1-receptor is present on melanocytes and melanoma cells (Low et al. 1994, Siegrist & Eberle 1995). Recent data also indicate that the MC1-receptor is expressed in limited areas (periaqueductal gray) of rat and human brain (Xia et al. 1995), as well as in the testis (Vanetti et al. 1994). Also, very importantly recently the MC1-receptor is shown to be present on macrophages (Star et al. 1995), neutrophils (Catania et al. 1996), glioma cells and astrocytes (Wong et al. 1997), monocytes and endothelial cells (Hartmeyer et al. 1997, and references therein). Recently the MC1-receptor mRNA was also detected in the normal mouse brain (Rajora et al. 1997a), suggesting a specific function of this receptor in the brain. Moreover, recent data obtained by immunohistochemical detection techniques show that MC1-receptors are present in testis and ovary (Thörmvall et al. 1997). This suggests a specific role of the MC1-receptor in reproductive physiology, as well as possibly in reproductive pathophysiology.

10 The MC2-receptor is the ACTH receptor. It is present in the cortex of the adrenal gland. The MC3-receptor mRNA is found in distinct areas of the brain, as well as in placental and gut tissues (Gantz et al. 1993a, Desarnaud et al. 1994, Roselli-Rehfuss et al. 1993). The MC4-receptor is ubiquitous in the brain (Gantz et al. 1993b, Mountjoy et al. 1994). The MC5-receptor is expressed in the brain, as well as in several peripheral tissues (Chhajlani et al. 1993, Gantz et al. 1994, Griffon et al. 1994, Labbé et al. 1994, Barrett et al. 1994, Fathi et al. 1995). More recent data indicate that all the 5 cloned MC-receptors have a wider tissue distribution (Chhajlani 1996).

15 The five MC-receptors show unique affinities for the melanocortin peptides (Schiöth et al. 1995, Schiöth et al. 1996a,b,c). Thus, the MC1-receptor shows high affinity for  $\alpha$ -MSH, but lower affinities for  $\beta$ -MSH,  $\gamma$ -MSH and ACTH. The MC2-receptor binds ACTH with high affinity, but it does not bind the MSH peptides. The MC3-receptor shows slightly higher affinity for  $\gamma$ -MSH compared to  $\beta$ - and  $\alpha$ -MSH. The MC4-receptor shows slight preference for  $\beta$ -MSH, over  $\alpha$ -MSH, and a very low affinity for  $\gamma$ -MSH. The MC5-receptor shows the same potency order for the MSH peptides as the MC1-receptor, although with much lower affinities (Schiöth et al. 1995, Schiöth et al. 1996a,b,c).

20 A diversity of effects is induced by natural melanocortin peptides not yet fully related to the various MC-receptor subtypes. These effects should be mediated by different subtypes of the MC-receptors. Most pertinent, however, is that increasing evidence indicates the MC1-receptors play an important role in the modulation of inflammation. Thus, for example,  $\alpha$ -MSH was shown to inhibit formation of nitric oxide (NO) in cultured murine macrophages

stimulated with bacterial lipopolysaccharide and  $\gamma$ -interferon, an effect claimed to be caused by the inhibition of the production of NO synthase (NOS) by the stimulation of MC1-receptors in macrophages (Star et al. 1995). As NO is believed to be a common mediator of all forms of inflammation this indicates that stimulation of MC1-receptors  
5 mediates the anti-inflammatory effect earlier known to be induced by MSH-peptides.  $\alpha$ -MSH is also known to increase the formation of interleukin 10 (IL-10) in monocytes, which is believed to be an important component in immunosuppressive effects induced by  $\alpha$ -MSH (Bhardwaj et al. 1996).

10 Recent studies also show that  $\alpha$ -MSH potently inhibits the chemotactic migration of neutrophils (Catania et al. 1996). Moreover, neutrophils were shown to contain MC1-receptor mRNA, which was upregulated on stimulation of the neutrophils with interferon and bacterial lipopolysaccharide (Catania et al. 1996). Thus, as neutrophil migration constitutes an important component in early inflammation, these results again indicate the importance  
15 of the MC1-receptor as mediators of inhibition of inflammation.

In another study the injection of  $\alpha$ -MSH, as well as the MSH-analogue [Nle<sup>4</sup>,D-Phe<sup>7</sup>] $\alpha$ -MSH (NDP-MSH) was shown to inhibit the release of cytokines IL-1 and TNF- $\alpha$  into the blood after intra-peritoneal injection of lipopolysaccharide (Goninard et al. 1996). This  
20 supports the anti-inflammatory role of MSH-peptides.

Important anti-inflammatory roles of MC-receptors (presumed to be of the MC1 type) have also been implicated in the brain since  $\alpha$ -MSH inhibits the production of tumour necrosis factor alpha (TNF- $\alpha$ ) *in vivo*, as well as *in vitro* on glioma cells; in the later case  $\alpha$ -MSH was  
25 shown to inhibit formation of TNF- $\alpha$  induced by bacterial endotoxin (Wong et al. 1997). In another study  $\alpha$ -MSH given intracerebroventricularly or intraperitoneally inhibited formation of central TNF- $\alpha$  induced by locally administered bacterial lipopolysaccharide (Rajora et al. 1997a). TNF- $\alpha$  occurs in neurological disorders, infection and injury of the brain, and is thought to underlie pathological processes in the brain. These data indicate an important  
30 role of MC-receptors as mediators of central anti-inflammatory actions.

Recently  $\alpha$ -MSH was also shown to reduce inflammation in a model for inflammatory bowel disease (Rajora et al. 1997b).

The  $\alpha$ -MSH peptide too is ascribed an important role in cutaneous biology. Most well known is its ability to stimulate pigment formation of the skin.  $\alpha$ -MSH may act not only on MC-receptors located to melanocytes but also on immunocompetent and inflammatory cells, keratinocytes, fibroblasts and endothelial cells of the skin, thereby modifying

5 keratinocyte proliferation and differentiation, and regulate endothelial cell and fibroblast cytokine production, as well as fibroblast collagenase production.  $\alpha$ -MSH is known to down-regulate the production of pro-inflammatory cytokines and accessory molecules on antigen presenting cells. In contrast suppressor factors such as IL-10 are upregulated by  $\alpha$ -MSH (Luger 1997). *In vivo* data show that systemic application of  $\alpha$ -MSH inhibits the induction

10 and elicitation of contact hypersensitivity and induces hapten tolerance (Luger 1997). Thus, the accumulating evidence indicates that the stimulation of MC-receptors, presumably of the MC1-receptor subtype, mediates important negative regulation mechanisms of cutaneous inflammation and hyper-proliferative skin diseases (Luger 1997).

15 In addition to these findings, Hartmeyer et al. (1997) have shown that  $\alpha$ -MSH increases MC1-receptor expression in dermal microvasculature endothelial cells and causes increased release of interleukin 8 (IL-8) from these cells. This indicates a role of MC1-receptors in the skin as modulators of inflammation and immunity (see Hartmeyer et al. 1997).

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For further reading on the anti-inflammatory role of MSH peptides reference is made to the review by Lipton and Catania (1997).

Since 1957 MSH-receptors have been known as physiological entities. Binding sites for

25 MSH/ACTH peptides have been identified in number of brain and peripheral tissues (Hnatowich et al. 1989, Tatro & Reichlin 1987, Lichtensteiger et al. 1993, Tatro & Entwistle 1994). Peptide structure activity studies of these receptors have been performed on melanophores from lower vertebrates like *Rana pipiens* (frog), *Anolis carolinensis* (lizard) and *Xenopus laevis*. Receptor studies were later also performed by binding on melanoma

30 cell lines. These test systems gave comparable results and it is now known that the data obtained with these systems refer to the MC1-receptor (Eberle 1988).

Using such test systems it was found that replacement of L-Phe by D-Phe in  $\alpha$ -MSH provided high potency and prolonged action (Sawyer et al., 1980). Cyclic [Cys<sup>4</sup>,  
35 Cys<sup>10</sup>] $\alpha$ -MSH analogues were also synthesized; they were found to be potent

melanotrophs in skin pigmentation bioassays (Knittle et al., 1983, Sawyer et al., 1982). However, while some of the previous known natural and synthetic peptides, as well as more recently synthesized ones, show high affinity for MC1-receptors, their selectivity versus other subtypes of MC-receptors are limited (see e.g. Hol et al. 1994, Adan et al. 1994, among others). Recently, however, a peptide found by phage display screening was described which showed higher selectivity for MC1-receptors compared to other subtypes for MC receptors (Szardenings et al. 1997). However, for clinical and other uses this peptide showed inferior properties due to 1) comparatively low affinity for MC1-receptors, 2) low agonistic effects on MC1-receptors compared to other peptide hormones and 3) low stability due to presence of oxidizable SH groups and instability in regard of proteolytic cleavage.

There remains a need to provide means and methods to selectively regulate MC1-receptors. Thereby pharmacological effects affecting processes and conditions related to tissues and cells expressing the MC1-receptor may be elicited. These processes and conditions comprise but are not limited to immune responses, inflammatory processes, immunotolerance, immunomodulation, allergic processes, reproductive processes, melanoma and malignant diseases related to MC1-receptor expressing cells.

There is furthermore a need to provide chemical compounds that activate MC1-receptors selectively and with high potency; to provide chemical compounds which antagonize the action of other hormones and agonists on MC1-receptors selectively and with high potency; and to provide a method for administration of said compounds to animals including man.

*Description of the Invention*  
According to the present invention are disclosed novel compounds showing high selectivity and high affinity for MC1-receptors in combination with effective stimulation of cAMP formation in MC1-receptor expressing cells. On the other hand the compounds of the invention show low or negligible affinity for other subtypes of MC-receptors.

According to the present invention are also disclosed novel compounds which inhibit the production of nitric oxide (NO).

According to the present invention are also disclosed novel compounds which are immunomodulatory and anti-inflammatory.

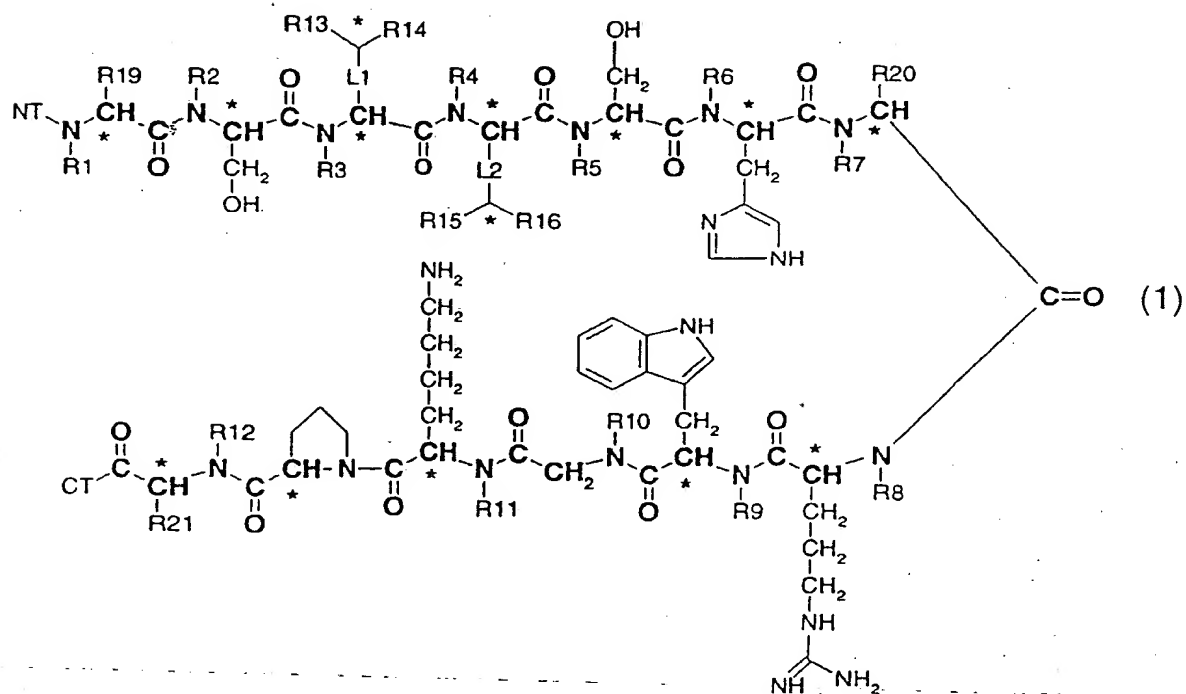
The compounds according to the present invention are represented by the general formula (1):

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wherein R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11 and R12 are selected independently from each other from H and methyl, with H being preferred,

and wherein R13, R14, R15 and R16 are selected independently from each other from H and alkyl, in particular alkyl selected from methyl, ethyl, propyl, isopropyl, and wherein optionally one hydrogen in R13 and one hydrogen in R14 is exchanged for a bond between R13 and R14, and wherein optionally one hydrogen in R15 and one hydrogen in R16 is

exchanged for a bond between R15 and R16, and wherein L1 and L2 are linkers which are independently selected from the group consisting of single bond, methyl, ethyl, with single bond being preferred,

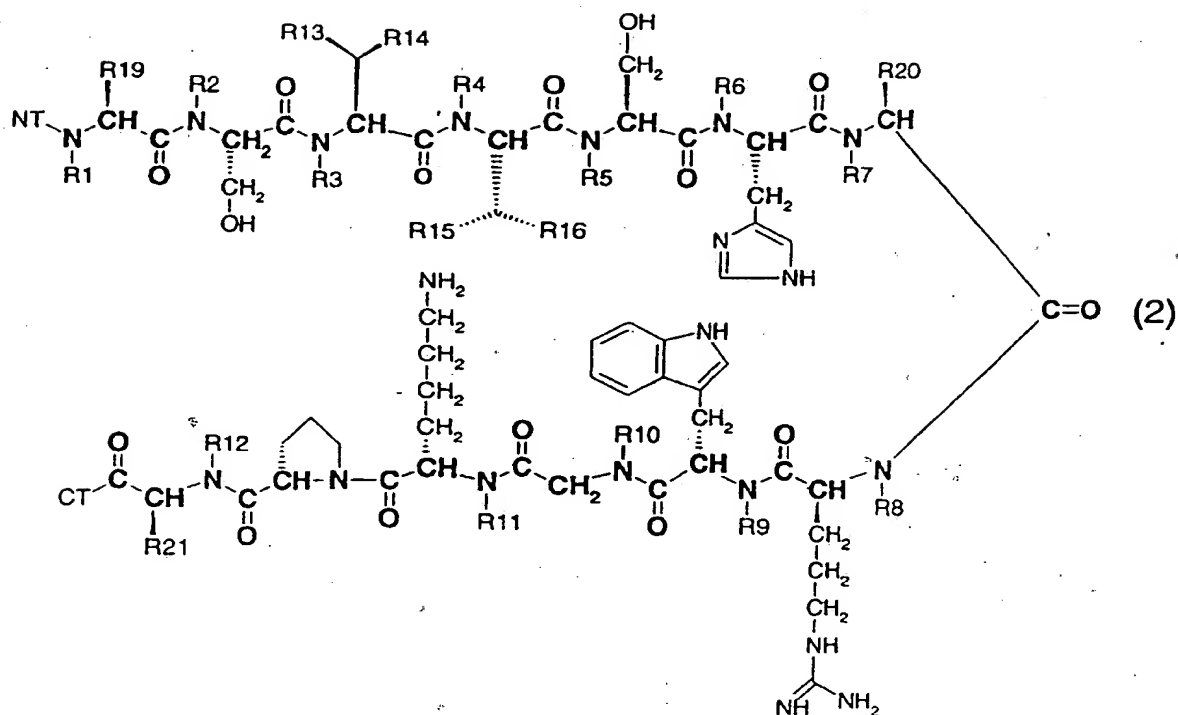
- 5 and wherein R19, R20 and R21 are selected independently from each other from H and -CH<sub>2</sub>X, where X is H, alkyl, substituted alkyl, heteroalkyl, substituted heteroalkyl, alkenyl, substituted alkenyl, heteroalkenyl, substituted heteroalkenyl, alkynyl, substituted alkynyl, heteroalkynyl, substituted heteroalkynyl, cycloalkyl, substituted cycloalkyl, cycloheteroalkyl, substituted cycloheteroalkyl, cycloalkenyl, substituted cycloalkenyl, cycloheteroalkenyl, substituted cycloheteroalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, functional group,
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- and wherein NT is selected from H, hydroxyl, alkyl, aminoacid, aminoacid analogue, polypeptide and functional group, and CT is selected from hydrogen, hydroxyl, alkyl, aminoacid, aminoacid analogue, polypeptide and functional group, and wherein each asymmetric center (\*) is in R or S configuration.
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Preferably R20 is phenyl.

- 20 Recognizing that compounds according to formula (1) represent peptides, it is furthermore understood that the compounds of the invention are not limited to peptides according to formula (1), but include also compounds wherein one or several of the nitrogens of the peptide backbone have been exchanged for a carbon substituted with hydrogen, and/or wherein one or several of the oxygens of the carbonyl groups of the peptide backbone has been exchanged for two hydrogens.
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The preferred stereomeric conformation of the compound (1) of the invention is disclosed in formula (2):



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wherein R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11 and R12 are selected independently from each other from H and methyl, with H being preferred,

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and wherein R13, R14, R15 and R16 are selected independently from each other from H and alkyl, in particular alkyl selected from methyl, ethyl, propyl, isopropyl, and wherein optionally one hydrogen in R13 and one hydrogen in R14 is exchanged for a bond between R13 and R14, and wherein optionally one hydrogen in R15 and one hydrogen in R16 is exchanged for a bond between R15 and R16,

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and wherein R19, R20 and R21 are selected independently from each other from H and -CH<sub>2</sub>X, where X is H, alkyl, substituted alkyl, heteroalkyl, substituted heteroalkyl, alkenyl, substituted alkenyl, heteroalkenyl, substituted heteroalkenyl, alkynyl, substituted alkynyl, heteroalkynyl, substituted heteroalkynyl, cycloalkyl, substituted cycloalkyl, cycloheteroalkyl, substituted cycloheteroalkyl, cycloalkenyl, substituted cycloalkenyl, cycloheteroalkenyl, substituted cycloheteroalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, functional group,

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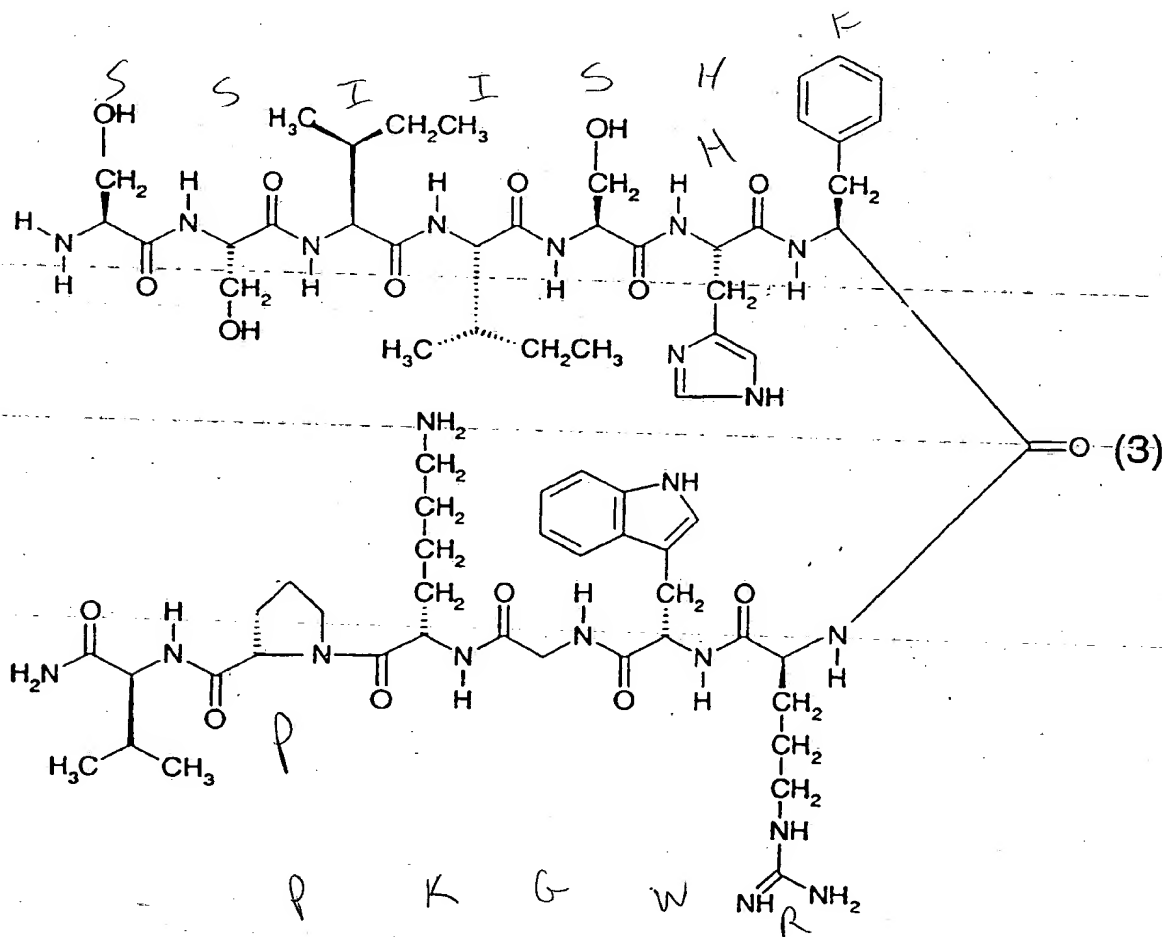
and wherein NT is selected from H, hydroxyl, alkyl, aminoacid, aminoacid analogue, polypeptide and functional group, and CT is selected from hydrogen, hydroxyl, alkyl, aminoacid, aminoacid analogue, polypeptide and functional group.

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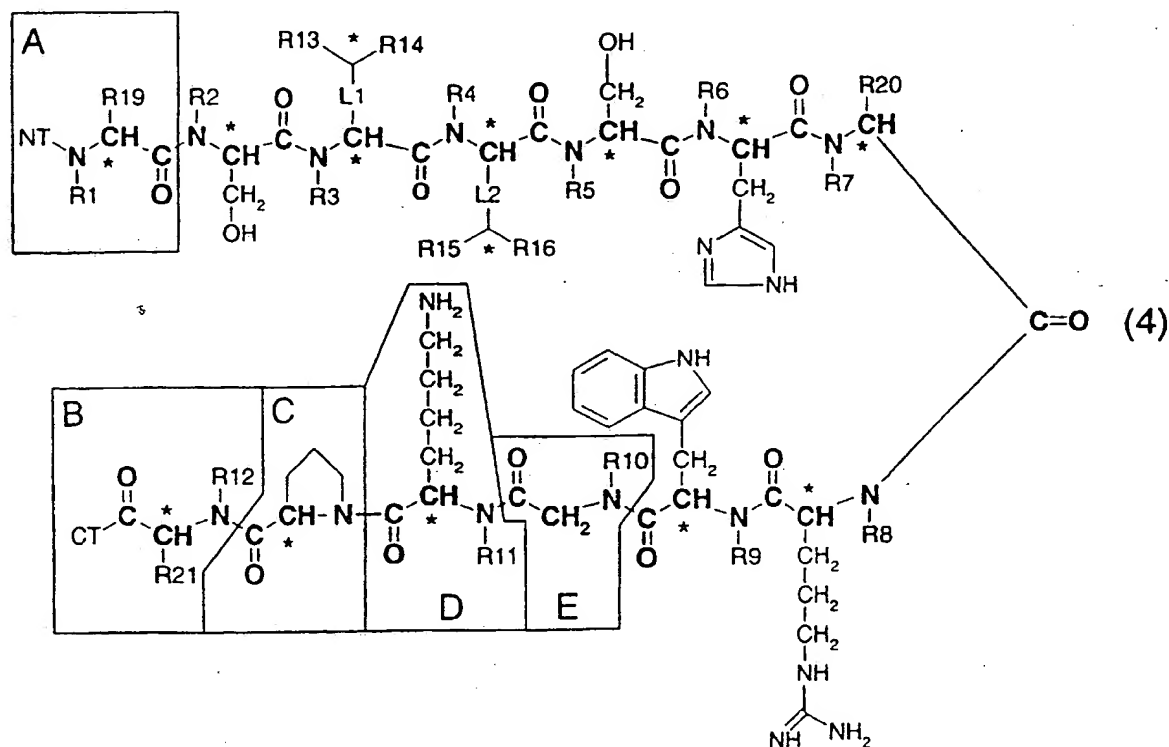
For the compound according to formula (2) the preferred substituent of R20 is phenyl.

Recognizing that compounds according to formula (2) represent peptides, it is furthermore understood that the compounds of the invention are not limited to peptides according to formula (2), but include also compounds wherein one or several of the nitrogens of the peptide backbone has been exchanged for a carbon substituted by hydrogen, and/or wherein one or several of the oxygens connected to carbonyl groups of the peptide backbone has been exchanged for two hydrogens.

15 According to the invention is disclosed a particularly preferred compound (3), which in the following will be termed MS05 (SEQ ID NO:1):



The invention also includes fragments of the compounds according to formula (1), (2) and (3), as detailed in the general formula (4) in which five moieties A, B, C, D, E have been delimited:



wherein R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11 and R12 are selected independently from each other from H and methyl, with H being preferred,

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and wherein R13, R14, R15 and R16 are selected independently from each other from H and alkyl, in particular alkyl selected from methyl, ethyl, propyl, isopropyl, and wherein optionally one hydrogen in R13 and one hydrogen in R14 is exchanged for a bond between R13 and R14, and wherein optionally one hydrogen in R15 and one hydrogen in R16 is exchanged for a bond between R15 and R16, and wherein L1 and L2 are linkers which are independently selected from the group consisting of single bond, methyl, ethyl, with single bond being preferred,

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and wherein R19, R20 and R21 are selected independently from each other from H and -CH<sub>2</sub>X, where X is H, alkyl, substituted alkyl, heteroalkyl, substituted heteroalkyl, alkenyl, substituted alkenyl, heteroalkenyl, substituted heteroalkenyl, alkynyl, substituted alkynyl,

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heteroalkynyl, substituted heteroalkynyl, cycloalkyl, substituted cycloalkyl, cycloheteroalkyl, substituted cycloheteroalkyl, cycloalkenyl, substituted cycloalkenyl, cycloheteroalkenyl, substituted cycloheteroalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, functional group,

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and wherein NT is selected from H, hydroxyl, alkyl, aminoacid, aminoacid analogue, polypeptide or functional group, and CT is selected from hydrogen, hydroxyl, alkyl, aminoacid, aminoacid analogue, polypeptide, functional group, and wherein each asymmetric center (\*) is in R or S configuration,

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and wherein moiety A is optionally exchanged for hydrogen, hydroxyl, alkyl, aminoacid, aminoacid analogue, polypeptide, or a functional group,

and wherein moiety B is optionally exchanged for hydrogen, hydroxyl, alkyl, aminoacid, aminoacid analogue, polypeptide, or a functional group,

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and wherein optionally moiety C is exchanged for aminoacid or aminoacid analogue,

and wherein optionally moiety D is exchanged for aminoacid or aminoacid analogue,

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and wherein optionally moiety E is exchanged for aminoacid or aminoacid analogue.

In a further advantageous embodiment of the invention according to formula (4) moiety C is exchanged for aminoacid or aminoacid analogue, whereas moieties D and E are retained.

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In a further advantageous embodiment of the invention according to formula (4) moiety D is exchanged for aminoacid or aminoacid analogue, whereas moieties D and E are retained.

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In a still further advantageous embodiment of the invention to formula (4), as defined above, moiety E is exchanged for aminoacid or aminoacid analogue, whereas the moieties C and D are retained.

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In certain preferred embodiments of the invention one or several of R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11 and R12 of the compounds according to formulae (1), (2) or (4) are selected to be methyl, whereas the rest is selected to be hydrogen, the selections being

made so as to prevent or decelerate breakdown by proteases and/or peptidases. Moreover, the peptide backbone of the compounds according (1), (2) and (4) may be modified by exchanging carbon, nitrogen and oxygen atoms by other atom(s), preferably oxygen, carbon and hydrogen, respectively, so as to prevent or decelerate breakdown by proteases and/or peptidases.

In even more advantageous embodiments of the invention less than 6, preferably less than 5, more preferred less than 4 and preferably less than 2, and most preferred none of the R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11 and R12 in compounds according to formula (1), (2) and (4) are selected to be methyl.

Moreover the peptide backbone of the compounds according to formula (1), (2) and (4) may be modified by exchanging carbon, nitrogen and oxygen atoms by other atom(s) so as to prevent or decelerate breakdown by proteases or peptidases, the preferred substitute for carbon being oxygen, for nitrogen being carbon, and for oxygen, hydrogen. In a preferred embodiment of this aspect of the invention preferably less than 5, more preferably less than 4, even more preferably less than 3, and most preferred less than 1 of said peptide backbone carbon, nitrogen and oxygen atoms are exchanged for oxygen, carbon or hydrogen.

In the present specification the term "peptide backbone" refers to the atoms marked by their bold characters in formulae (1), (2) and (4).

In addition R19, R20 and R21 may be selected so as to provide a modified compound the digestion of which by proteases or peptidases is delayed or prevented.

Included in the invention is a compound of formula (1), (2), (3) or (4) which has been subjected to minor structural alteration by the exchange of not more than five, preferably not more than four, more preferred not more than three, even more preferred not more than two and most preferred not more than one by methyl.

The invention comprises salts of the compound of the invention by known acids, including the trifluoroacetate, pharmaceutically acceptable salts being preferred.

In a preferred embodiment of the invention a rapid breakdown of the compound of the invention by endogenous enzymes is desired as this will provide a medicine short half life. It

is however recognized that such a compound may act as a "hit-and-run" drug, the biological action of which extends beyond the period of time within which the presence of the compound of the invention can be detected in the respective animal; this effect due to a prolonged activation of the MC1- receptor.

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It is also recognized that the structural variations of the compound of invention, as disclosed above, in particular the proper selection of substituents R19, R20, and R21, can be made so as to provide compounds with a capacity to either selectively activate or selectively block an MC1-receptor. Typical activation of MC1-receptor by a compound of the invention leads to stimulation of the generation of second messenger element cAMP, as shown in Example 3, as well as biological responses and pharmacological effects related to the MC1-receptor. Activation of the MC1-receptor may also lead to receptor desensitization and/or receptor down regulation, something which in turn may lead to a decreased capacity of the MC1-receptor to generate biological responses and/or accumulation of cAMP. In some embodiments of the invention the capacity to afford MC1-receptor desensitization and/or MC1-receptor down regulation is a desired effect. Moreover, in further embodiments of the invention upregulation and/or increased expression of the MC1-receptor can be afforded by the administration of the compound of the invention, which may also be a highly desirable effect.

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On the other hand typical blockade by a compound of the invention of an MC1-receptor will prevent the induction of biological response by an endogenous or exogenous MSH peptide, a peptide analogue or a MC1-receptor stimulatory compound.

25 Compounds according to the invention typically showing capacity to activate MC1-receptors are represented by (but not limited to) compounds complying with formula (1), (2) or (4) in which R20 is aryl, substituted aryl, heteroaryl, substituted heteroaryl, phenyl or substituted phenyl. Compounds according to the invention typically showing capacity to block MC1-receptors are represented by (but not limited to) compounds complying with formula (1), (2) or (4) in which R20 is aryl, substituted aryl, heteroaryl, substituted heteroaryl, naphthalene, 30 or substituted naphthalene.

Further embodiments of the invention are directed to compounds decreasing the formation of interleukin 1 (IL-1), interleukin 6 (IL-6), and tumour necrosis factor  $\alpha$  (TNF-  $\alpha$ ), to afford 35 decreased production of nitric oxide and downregulate the activity of nitric oxide synthase (NOS). Other embodiments of the invention are directed to compounds stimulating the

production of interleukin 8 (IL-8) and/or interleukin 10 (IL-10). Yet other embodiments of the invention are directed to producing an effect opposite to that described in regard of IL-1, IL-6, TNF- $\alpha$ , nitric oxide, NOS, IL-8 and IL-10.

- 5 The compounds of the invention may be manufactured by conventional chemical techniques known in the art. They are exemplified the synthesis of MS05 described in Example 1.1.

10 The compounds of the invention may also be manufactured by conventional molecular biological techniques known in the art. In this case the DNA-sequence encoding a peptide complying with formula (1), (2), (3) or (4) is typically manufactured first. The thus obtained DNA then is incorporated into an expression vector for use in a conventional expression system. Suitable expression systems are also known in the art and comprise eukaryotic or prokaryotic cells. Due to this possibility of manufacture of the compounds of the invention  
15 using molecular biological techniques, the present invention also includes DNA encoding a compound of the invention. The DNA encoding the compound of the invention may be joined at one or both of its 3' and 5'-ends to other DNA. The invention therefore includes DNA comprising a DNA sequence encoding the compound of the invention. The DNA encoding the compound of the invention may be present in a vector where it is joined with  
20 other DNA at one or both of its 3' and 5'-ends. The invention therefore includes a vector which comprises DNA encoding the compound of the invention.

The present invention also includes a fusion protein comprising one or several copies of the sequence of a compound of the invention. Such a fusion protein is typically manufactured  
25 by use of an expression system by utilizing the proper DNA, according to the principles described above, and by application of procedures well known in the art.

The invention also includes a vector comprising a DNA which encodes a fusion protein comprising the amino acid sequence of one or several copies of the compound of the  
30 invention. According to the present invention DNA can be exchanged for a chemically altered non-natural DNA, said non-natural DNA being capable of essentially affording the same function as natural DNA with respect to peptide or protein synthesis. Moreover, the DNA according to the invention can also be exchanged for RNA. It is particularly advantageous to use RNA or a non-natural RNA when the non-natural DNA or RNA is  
35 administered to an animal, in particular a human, the reason being avoidance of RNA or

non-natural DNA recombining with endogenous DNA of the animal or human, thus diminishing the risk of long term side effects.

5 The term "alkyl" as employed herein by itself or as part of another group includes a straight or branched hydrocarbon chain of up to 18, preferably from 1 to 8 carbon atoms, such as methyl, ethyl, propyl, isopropyl, tert-butyl, butyl, pentyl, hexyl, heptyl or octyl.

10 The term "heteroalkyl" as employed herein by itself or as part of another group refers to alkyl wherein one or several carbon atoms are exchanged for heteroatom.

15 The term "alkenyl" as employed herein by itself or as part of another group includes a straight or branched hydrocarbon chain of up to 18, preferably from 2 to 8 carbon atoms comprising one or several carbon-carbon double bonds, such as propenyl, butenyl, pentenyl.

20 The term "heteroalkenyl" as employed herein by itself or as part of another group refers to alkenyl wherein one or several carbon atoms are exchanged for heteroatom.

The term "alkynyl" as employed herein by itself or as part of another group refers to alkyl or alkenyl containing one or several carbon-carbon triple bonds.

The term "heteroalkynyl" as employed herein by itself or as part of another group refers to heteroalkyl or heteroalkenyl containing one or several carbon-carbon triple bonds.

25 The term "cycloalkyl" as employed herein by itself or as part of another group refers to cyclic hydrocarbons containing from 3 to 12 carbons, preferably 3 to 8 carbons, such as cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, which may optionally be fused with 1 or 2 cycles, each cycle being independently selected from the group consisting of cycloalkyl, cycloheteroalkyl, cycloalkenyl, cycloheteroalkenyl, aryl  
30 and/or heteroaryl.

The term "cycloheteroalkyl" as employed herein by itself or as part of another group refers to cycloalkyl where one or several carbon atoms are exchanged for heteroatom.

The term "cycloalkenyl" as employed herein by itself or as part of another group refers to cycloalkyl containing one or several carbon-carbon double bonds, such as cyclopentenyl and cyclohexenyl.

- 5 The term "cycloheteroalkenyl" as employed herein by itself or as part of another group refers to cycloheteroalkyl where one or more bonds between carbons, carbon and heteroatom, or heteroatoms are double.

- 10 The term "aryl" as employed herein by itself or as part of another group refers to phenyl which may optionally be fused with 1 or 2 cycles which are independently selected from cycloalkyl, cycloheteroalkyl, cycloalkenyl, cycloheteroalkenyl, aryl, heteroaryl.

- 15 The term "heteroaryl" as employed herein by itself or as part of another group refers to a 5- to 12-membered aromatic ring, preferably 5- to 6-membered aromatic ring, which includes one or more heteroatoms, and which may optionally be fused with 1 or 2 cycles which are independently selected from cycloalkyl, cycloheteroalkyl, cycloalkenyl, cycloheteroalkenyl, aryl, heteroaryl.

- 20 The term "substituted" refers to one or more hydrogens being substituted, independently of each other, by alkyl, fluorinated alkyl, alkenyl, fluorinated alkenyl, alkynyl, fluorinated alkynyl, cycloalkyl, fluorinated cycloalkyl, cycloheteroalkyl, fluorinated cycloheteroalkyl, cycloalkenyl, fluorinated cycloalkenyl, cycloheteroalkenyl, fluorinated cycloheteroalkenyl, aryl, fluorinated aryl, heteroaryl, fluorinated heteroaryl, functional group. Moreover, if a structure connected with the term "substituted" is a cyclic structure fused with another cyclic structure or other cyclic structures, the latter cyclic structure(s) may also be substituted.
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The term "halogen" as employed herein by itself or a part of another group refers to chlorine, bromine, fluorine and iodine, with chlorine being preferred.

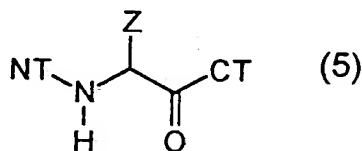
- 30 The term "heteroatom" as employed herein by itself or as part of another group refers to nitrogen, oxygen or sulfur, to which one or more hydrogens may be connected according to its valence, and in which in the case of nitrogen one oxygen atom may be optionally connected to it by donor acceptor bond, thus forming N-oxide.
- 35 The term "functional group" as employed herein by itself or as part of another group refers to amino, alkylamino, dialkylamino, arylamino, arylazido, heteroarylamino, heteroarylazido,



hydroxy, alkylhydroxy, fluorinated alkylhydroxy, cyano, carboxy, alkylcarboxy, arylcarboxy, halogen, nitro, hydroxylamino, acyl, fluorinated acyl, nitroso, sulfonyl, sulfinyl, thio, alkylthio, arylthio.

- 5 The term "fused" as employed herein by itself or as part of another group refers to two or three cycles having one or more common atoms, the preferred maximum number of fused cycles being three.

- The term "aminoacid" as employed herein by itself or as part of another group refers to
- 10 alanine, arginine, asparagine, aspartic acid, p-benzoyl-phenylalanine,  $\beta$ -cyclohexyl-alanine, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine,  $\beta$ -(2-naphtyl)-alanine, norleucine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, 3,4-dichlorophenylalanine, 4-fluorophenylalanine, 4-nitrophenylalanine, 2-thienylalanine, 3-benzothierylalanine, 4-cyanophenylalanine, 4-iodophenylalanine, 4-
- 15 bromophenylalanine, 4,4'-biphenylalanine, pentafluorophenylalanine,  $\beta$ ,  $\beta$ -diphenylalanine, in either D- or L-conformations, D-L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, as well as other substances having the following general structure (5):

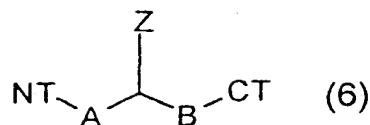


20

- in which Z is H or  $-\text{CH}_2\text{X}$ , where X is H, alkyl, substituted alkyl, heteroalkyl, substituted heteroalkyl, alkenyl, substituted alkenyl, heteroalkenyl, substituted heteroalkenyl, alkynyl, substituted alkynyl, heteroalkynyl, substituted heteroalkynyl, cycloalkyl, substituted cycloalkyl, cycloheteroalkyl, substituted cycloheteroalkyl, cycloalkenyl, substituted
- 25 cycloalkenyl, cycloheteroalkenyl, substituted cycloheteroalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, or functional group, NT is H, functional group or bond to another aminoacid, and CT is functional group or bond to another aminoacid, the compound according to formula (5) being in either D- or L-conformation.

- 30 The term "aminoacid analogue" as employed herein by itself or as part of another group refers to a substance having the following general structure (6):

18



wherein A is oxygen, nitrogen or carbon to which hydrogen and/or methyl is attached according to valence,

and wherein B is oxygen or carbon to which hydrogen and/or oxygen is attached according to valence,

and wherein each asymmetric centre is in either R or S configuration,

and wherein Z is H or -CH<sub>2</sub>X, where X is H, alkyl, substituted alkyl, heteroalkyl, substituted heteroalkyl, alkenyl, substituted alkenyl, heteroalkenyl, substituted heteroalkenyl, alkynyl, substituted alkynyl, heteroalkynyl, substituted heteroalkynyl, cycloalkyl, substituted cycloalkyl, cycloheteroalkyl, substituted cycloheteroalkyl, cycloalkenyl, substituted cycloalkenyl, cycloheteroalkenyl, substituted cycloheteroalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, or functional group, NT is H, functional group, bond to another aminoacid, or bond to another aminoacid analogue, and CT is functional group, bond to another aminoacid, or bond to another aminoacid analogue.

The compounds of the present invention have unique novel properties constituting their capacity to bind preferentially to the MC1-receptor among the five known subtypes of MC-receptors. This capacity is revealed in tests on recombinant human MC-receptors, using methods described in the literature (see Schiöth et al., Eur. J. Pharmacol., Mol. Pharm. Sect. 1995, 288, 311 and Pharmacol. & Toxicol. 1996, 79, 161). In these tests the capacity of the compounds to compete for the binding of [<sup>125</sup>I]-labelled NDP-MSH ([Nle<sup>4</sup>, D-Phe<sup>7</sup>]<sub>α</sub>-MSH) to recombinant human MC-receptor subtypes expressed in COS-1 cells was evaluated, as described in Example 2. With these assays the following dissociation constants (K<sub>i</sub>s) were determined for some of the compounds of the invention:

		<u>MC1</u>	<u>MC3</u>	<u>MC4</u>	<u>MC5</u>
	<u>Compound</u>	<u>K<sub>i</sub> (nM)</u>	<u>K<sub>i</sub> (nM)</u>	<u>K<sub>i</sub> (nM)</u>	<u>K<sub>i</sub> (nM)</u>
5	NDP-MSH	0.11	0.39	2.3	2.9
	$\alpha$ -MSH	0.68	52.3	2030	4990
10	MS05	0.76	1365	>>50000	>>50000
	MS09	0.20	7.0	40	120

15 In the above table the K<sub>i</sub>-values for non-labeled NDP-MSH and the natural  $\alpha$ -MSH-peptide is given for comparison. It is evident from the Table that MS05, which is a particularly preferred compound of the invention, shows the same high affinity for the MC1-receptor as  $\alpha$ -MSH, whereas it shows 1800-fold lower affinity for the MC3 receptor, and more than 65000-fold lower affinities for the MC4 and MC5 receptors, respectively.

20 (In the above table >>50000 is intended to mean "larger than 50000" which means that MS05 did not show any appreciable capacity to bind to either the MC4- or MC5-receptor in concentrations up to 50000 nM). MS05 thereby shows very high selectivity for MC1-receptors when compared to its affinity for MC3, MC4 and MC5 receptors. Moreover, MS05 has very high affinity for the MC1-receptor. As is evident from the Table, MS09 is another  
 25 preferred compound of the invention as it shows extremely high affinity for the MC-1 receptor. These properties are combined with an increased stability in comparison with compounds known in the art, something which seems to be due to the lack of easily oxidizable groups (e.g. -SH groups and -SH-CH<sub>3</sub> groups such as in cysteine and methionine). In addition MS05 and MS09 show a higher capacity to stimulate the  
 30 generation of the second messenger cAMP compared to previously known compounds (see Example 3). The combination of the very high selectivity of MS05 and MS09 with their very high selectivity and good ability to stimulate formation of cAMP make MS05 and MS09 noteworthy. In Examples 3, 5 and 7 the high capacity of MS05 and MS09 to induce effective stimulation of cAMP is demonstrated.

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Examples 4-7 show the capacity of the compounds of the invention to bind to native MSH-receptors (i.e. native MC1 receptors) in melanoma cells and macrophages, as well as to induce stimulation of cAMP in these cells. Example 8 demonstrates the capacity of the compounds of the invention to inhibit nitric oxide (NO) formation in macrophages where NO

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production had been stimulated by inflammatory agents (bacterial lipopolysaccharide and interferon gamma). The results show that the compounds of the invention are very effective inhibitors of NO which in a further sense demonstrates that the compounds of the invention are effective anti-inflammatory agents.

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In the present specification by the term "high capacity to stimulate the second messenger cAMP" refers to the ability of 10 nM of a compound of the invention to induce at least 35 %, preferably at least 45 %, more preferred at least 55 %, most preferred at least 65 % stimulation of cAMP-formation in the MC1-receptor expressing cells described in Example 3, in comparison to that induced by 10 nM of  $\alpha$ -MSH in the same system, using the method described in Example 3.

10

However, in the present specification a preferred method to determine whether or not a compound ("compound" is in this context also mutatis mutandis termed "test compound") shows a "high capacity to stimulate the second messenger cAMP" constitutes contacting a Specified\_Concentration\_A of the test compound to B16-F1 mouse melanoma cells (American Type Culture Collection) for a period of between 10 to 60 min, with a period of between 10 - 30 min being more preferred, and with a period of 20 min being most preferred, essentially using the procedures described in Example 5, and then determining the amount of cAMP formed in the cells. By a "high capacity to stimulate cAMP", according to this procedure, is defined a stimulation of cAMP amounting to at least 30 %, more preferably amounting to at least 40 %, somewhat more preferably amounting to at least 50%, even somewhat more preferably amounting to at least 60 %, still even more preferably amounting to at least 70 %, even still even more preferably amounting to at least 80 %, and most preferably amounting to at least 90 %, of that induced by contacting Specified\_Concentration\_A of  $\alpha$ -MSH to B16-F1 mouse melanoma cells, using the same procedure as for the test compound. By "stimulation of cAMP" is in this context intended the increased level of cAMP induced over the basal level of cAMP, the basal level of cAMP being defined as the level of cAMP being present in the B16-F1 cells which have been subjected to the identical procedures as when cells were contacted with test compound, except that no test compound or  $\alpha$ -MSH is present during the period allocated for contacting. According to this procedure for determining the capacity of the test compound to stimulate cAMP by "Specified\_Concentration\_A" is intended a single concentration selected from within the range 1 - 100 nM, with 1 nM being preferred, 100 nM even more preferred and 10 nM most preferred.

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By cAMP is in this context intended cyclic adenosine 3',5'-monophosphate (cyclic AMP).

As pointed out above the compounds of the invention are effective inhibitors of NO production. In the present specification a preferred method to determine whether or not a compound ("compound" is in this context also *mutatis mutandis* termed "test compound") is an "effective inhibitor of NO production" constitutes contacting Specified\_Concentration\_B of the test compound to mouse RAW 264.7 macrophage cells (American Type Culture Collection), the cells being concomitantly stimulated for their production of NO by the addition of between 0 - 300 ng/mL of bacterial lipopolysaccharide, with 50 -150 ng/mL of bacterial lipopolysaccharide being more preferred, and with 100ng/mL of bacterial lipopolysaccharide being most preferred, and the cells also being concomitantly stimulated for their production of NO by the addition of between 0 - 10 units/mL of mouse recombinant interferon gamma, with 3 - 7 units/mL of mouse recombinant interferon gamma being more preferred, and with 5 units/mL of mouse recombinant interferon gamma being most preferred, for a period of 1 - 22 hours, with 10 - 20 hrs being more preferred, and with 16 hours being most preferred, essentially using the procedures described in Example 8, and then determining the amount of nitric oxide (NO) formed, the NO being determined by using any method well known in the art, with the method described in Example 8, where the formation of NO is determined indirectly by measuring the amount of nitrite formed, being most preferred.

By an effective inhibitor of NO production, according to this procedure, is defined an inhibition of NO production amounting to at least 30 %, more preferably amounting to at least 40 %, somewhat more preferably amounting to at least 50 %, even somewhat more preferably amounting to at least 60 %, still even more preferably amounting to at least 70 %, even still even more preferably amounting to at least 80 %, and most preferably amounting to at least 90 %, of the inhibition of NO production afforded by contacting Specified\_Concentration\_B of  $\alpha$ -MSH to the mouse RAW 264.7 macrophage cells, using the same procedure as for the test compound. By "inhibition of NO production" is in this context intended the reduction of NO production from the level of NO production obtained in RAW 264.7 cells which have been subjected to the identical procedures as when cells were contacted with test compound, except that no test compound or  $\alpha$ -MSH is present during the period allocated for contacting. According to this procedure for determining the capacity of the test compound to inhibit NO production by "Specified\_Concentration\_B" is intended a single concentration selected

from within the range 0.1 - 100 nM, with 0.1 nM being preferred, 100 nM somewhat more preferred, 1 nM even more preferred, and 10 nM most preferred.

In addition many of the compounds of the invention, in particular many of those complying with formula (1) and formula (2), including the compound according to formula (4), show increased stability against breakdown by peptidases while retaining their high selectivity and binding affinity for MC1-receptor. These properties are particularly valuable if prolonged half life in the body after systemic administration, is desired, and for causing sustained pharmacological actions in a living animal, in particular a human.

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In addition, the compounds of the invention do not bind to MC2-receptors to an important extent which is of advantage.

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In the present specification, "MC1/MC3-selectivity" is defined as the ratio of the  $K_i$  of a compound for an MC3-receptor ( $K_i$ -MC3) over the  $K_i$  of the same compound for the MC1-receptor ( $K_i$ -MC1), the  $K_i$  values being measured as described in Example 2, using the method described by Schiöth et al. 1995 and 1996b; hence:

20

$$\text{MC1/MC3-selectivity} = \frac{K_i\text{-MC3}}{K_i\text{-MC1}}$$

25

In the present specification, a "very high MC1/MC3-selectivity" of a compound of the invention refers to a MC1/MC3-selectivity of at least 60, preferably at least 100, more preferred of at least 200, even more preferred of at least 700, most preferred of at least 1000, even of at least 1200. However, in practically all of the embodiments of the present invention by a "very high MC1/MC3-selectivity" suffices an MC1/MC3-selectivity of at least 10, preferably at least 20, more preferably at least 30.

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In the present specification "MC1/MC4-selectivity" of a compound is defined as the ratio of the  $K_i$  of the compound for an MC4 receptor ( $K_i$ -MC4) over the  $K_i$  of the same compound for the MC1-receptor ( $K_i$ -MC1), the  $K_i$  values being measured as described in Example 2 by using the method described by Schiöth et al. 1995 and 1996b; hence:

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$$\text{MC1/MC4-selectivity} = \frac{K_i\text{-MC4}}{K_i\text{-MC1}}$$

- 5 In the present specification a "very high MC1/MC4-selectivity" of a compound of the invention refers to a MC1/MC4 selectivity of at least 300, preferably of at least 1000, more preferred at least 3000, even more preferred of at least 10000, most preferred of more than 20000 and even more than 30000 and even 50000. However, in practically all of the embodiments of the present invention by a "very high MC1/MC4-selectivity" suffices an
- 10 MC1/MC4-selectivity of at least 30, preferably at least 70, more preferably at least 150.

- In the present specification "MC1/MC5-selectivity" of a compound is defined as the ratio of the  $K_i$  of the compound for an MC5 receptor ( $K_i\text{-MC5}$ ) over the  $K_i$  of the same compound for the MC1-receptor ( $K_i\text{-MC1}$ ), the  $K_i$  values being measured as described in Example 2,
- 15 using the method described by Schiöth et al. 1995 and 1996b; hence:

$$\text{MC1/MC5-selectivity} = \frac{K_i\text{-MC5}}{K_i\text{-MC1}}$$

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- In the present specification a "very high MC1/MC5-selectivity" of a compound of the invention refers to a MC1/MC5 selectivity of at least 300, preferably of more than 1000, more preferred of at least 3000, even more preferred of at least 10000, most
- 25 preferred at least 20000 and even more than 30000 and 50000. However, in practically all of the embodiments of the present invention by a "very high MC1/MC5-selectivity" suffices an MC1/MC5-selectivity of at least 30, preferably at least 70, more preferably at least 150.

- In the present context a "very high affinity" of a compound of the invention for an MC1-receptor indicates it exhibits a  $K_i$  value of less than 30 nM, preferably of less than 15 nM, more preferred of less than 7 nM, even more preferred of less than 5 nM, most preferred of less than 3 nM, even less than 2 nM and less than 1 nM, the  $K_i$  value for the MC1-receptor being measured as described in Example 2, by using the method described by Schiöth et al. 1995 and 1996b.

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However, in the present context a "very high affinity" of a compound of the invention for an MC1-receptor is intended that it exhibits a  $K_i$  value of less than 30 nM, preferably of less than 15 nM, more preferred of less than 7 nM, even more preferred of less than 5 nM, most preferred of less than 3 nM, even less than 2 nM and less than 1 nM, the  $K_i$  value for the MC1-receptor being measured as described in Example 4 using mouse B16-F1 melanoma cells.

In the present context, whenever reference is made to the use of an MC1 receptor, or whenever reference is made to the human MC1 receptor, or to the MC1 receptor described by Chhajlani & Wikberg (FEBS Lett, 1992, 309, 417), or to the MC1 receptor used by Schioth et al. Eur. J. Pharmacol. Mol. Pharm Sect, 1995, 288, 311-317 or Schioth, et al., Pharmacol. Toxicol. 1996, 79, 161-165, is intended the MC1 receptor which is encoded by the DNA which is available from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), Braunschweig, Germany under the accession number DSM 7214, and which is described in the patent application no. PCT/DK93/00273 (WO94/04674).

However, in the present context, whenever reference to use of an MC1 receptor is made the receptor to be used can also be selected from a native MSH-receptor being present on melanoma cells of mammalian or human origin, without any matter of the present application being altered; the selection of the native MSH-receptor on B16-F1 melanoma cells being a preferred choice.

In the present context, whenever reference is made to an MSH-receptor is furthermore also intended a binding site which is capable of binding [ $^{125}$ I] NDP-MSH with a dissociation constant of 10 nM or lower, the dissociation constant being determined by using a radio-ligand binding procedure well known in the art, the preferred approach being described by Schioth et al. Eur. J. Pharmacol. Mol. Pharm Sect, 1995, 288, 311-317 or Schioth, et al. Pharmacol. Toxicol. 1996, 79, 161-165, with the extension that the MSH-receptor can be present in any natural or non-natural cell line of mammalian or human origin. Moreover, according to this definition of the MSH-receptor the [ $^{125}$ I] NDP-MSH binding can be largely prevented by the presence of between 0.1  $\mu$ M to 10  $\mu$ M of  $\alpha$ -MSH during the assaying procedure.

In the present context, whenever reference is made to mouse RAW 264.7 macrophage cells, the RAW 264.7 macrophage cells can be exchanged with any other cells of



mammalian or human origin that possesses MSH-receptors, and which NO production is inhibited by the addition of  $\alpha$ -MSH, without any matter of the present context being altered.

- 5 Particularly preferred compounds according to the invention comprised by one or several of the structural formulas (1), (2), (3), (4), and for which one, preferably two, more preferred three, most preferred four of the properties selected from the group high MC1/MC3-selectivity, high MC1/MC4-selectivity, high MC1/MC5-selectivity and high affinity for an MC1-receptor are combined. Moreover, an further preferred compound of the invention is  
10 one in which the said properties are combined with a high capacity to stimulate the second messenger, cAMP.

- By the term "high selectivity for MC1 receptors" is in the present context intended that a compound shows three, more preferably two, and most preferably one of the properties  
15 selected from the group of very high MC1/MC3-selectivity, very high MC1/MC4-selectivity, very high MC1/MC5-selectivity.

- However, a very preferred compound according to the invention is comprised by one or several of the structural formulas (1), (2), (3), (4), which compound is also an effective  
20 inhibitor of NO production.

- Marked positive treatment effects were found upon administration of MS05 to BALB/C mice. Animals were sensitized by injection of 2,4-dinitrofluorobenzene (DNFB). First 30 $\mu$ L of 0.5 % DNFB was administered to the shaved abdomen of the mice and  
25 after 4 days 10 $\mu$ L of 0.3 % DNFB was challenged to one paw, another paw being unchallenged and serving as a control. MS05 was administered, using a solution of MS05 in 0.9 % saline, intraperitoneally two hours before sensitization, and then the same dose was given intra-peritoneally for four consecutive days, each dose of MS05 amounting to 0.05, 0.25, 0.37, 0.5 and 0.75 mg/kg. These treatments with MS05 were  
30 found to inhibit paw oedema by 3, 5, 12, 39 and 10 %, respectively, compared to animals that were subjected to the same DNFB administration but which had not been given MS05. Moreover, in these tests the DNFB induced a marked increase in total blood white cell counts as well as marked increase in granulocyte counts of the blood, the increase in total white cell blood counts and granulocyte counts being essentially  
35 normalized by the MS05 treatment, the best effect being seen at 0.5 mg/kg of daily intraperitoneally administrations of MS05. The DNFB administration also increased the

count of lymphocytes/monocytes in the blood and this increase was essentially returned to normal levels upon the administration of MS05. Blood platelet counts was also increased in DNFB treated animals; the increase being returned towards the normal by administration of MS05. Similar reduction of the oedema induced by DNFB sensitization of the ears of BALB/c mice was seen upon intravenous injections of either MS05 or MS09, using solutions of MS05 and MS09 in 0.9 % saline, to the animals. Thus, these results show that a compound of the invention prevents, ameliorates, and/or inhibits contact hypersensitivity, sensitization by a hapten, and/or has a positive treatment effect on oedema. Moreover these results show that the compound of the invention is capable of normalizing blood cell counts. Therefore the compound of the invention is in a further sense immunomodulatory. Moreover, these results demonstrate the capacity of a compound of the invention to be administrated in form of a pharmaceutical.

In further tests the MS05 or MS09 was administered to human microvascular endothelial cells (HMEC-1 cells; Department of Health & Human Services, Centres for Disease Control and Prevention, Atlanta, GA 30333, USA) in vitro. The HMEC-1 cells responded with an upregulated expression of mRNA for adhesion molecules ICAM-1, VCAM and E-Selectin, as well as by upregulation of the ICAM-1, VCAM and E-Selectin proteins, upon administration of TNF  $\alpha$  (10 ng/mL). The administration of MS05 or MS09 (preferred concentrations of MS05 and MS09 being within 0.01 nM to 10  $\mu$ M, and preferred times for contacting the cells with MS05 or MS09 being 3 – 48 hours) led to inhibition of the upregulated expression of mRNA for adhesion molecules ICAM-1, VCAM and E-Selectin, as well as to inhibition of the upregulation of the ICAM-1, VCAM and E-Selectin proteins. These results demonstrate the capacity of the compound of the invention to be immunomodulatory, to be useful for the treatment of inflammation related to the vasculature, e.g. having positive treatment effects in vasculitis. In this context by ICAM-1 is intended intercellular adhesion molecule type 1, by VCAM is intended vascular adhesion molecule, and by E-Selectin is intended endothelial selectin (Sluiter et al, J. Cardiovasc. Pharmacol. 1993, 22 Suppl 4: S37-44; Elangbam et al., Vet. Pathol. 1997 Jan, 34(1): 61-73).

The compounds of the invention can be used for the treatment and diagnosis of diseases and disorders and/or pathological conditions, including preventive treatment, in an animal, in particular a mammal, but they are most preferably used for these purposes in man. Moreover, the compounds of the invention can be used also in healthy individuals and in healthy animals to change their physiological state.

5 In such treatment or diagnosis the compound of the invention is administered in form of a pharmaceutical composition further comprising a pharmaceutical acceptable carrier and, optionally, tableting agents, wetting agents, binders and fillers, preservatives, such as antioxidants and anti-microbial agents, buffers and salts. Preferred carriers comprise injection media, particularly water. The compositions are administered by any conventional route including the oral, enteral, rectal and parenteral routes. Parenteral routes comprise intravenous, intramuscular, subcutaneous and peritoneal injection. The compounds of the invention may also be administered by inhalation, as nasal spray, and topically on the skin. 10 They may also be administered epidurally, intrathecally and intracerebroventricularly.

15 In particular the pharmaceutical composition containing a pharmacologically effective amount of a compound of the invention is administered to an animal, in particular man, for alteration of physiological state, diagnosis, prevention or therapeutic treatment of diseases, in particular conditions which are positively affected by the stimulation of MC1-receptors or alternatively positively affected by blockade, and/or by downregulation, and/or by desensitization of MC1-receptors. Examples of such conditions include inflammation of any type and any origin. In particular is therewith intended inflammation or any related condition as well as any condition involving the action of macrophages, neutrophils, monocytes, 20 keratinocytes, fibroblasts, melanocytes, pigment cells and endothelial cells. Moreover included are conditions caused by or associated with increased production and/or release of inflammatory cytokines such as interleukins, in particular interleukin 1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ). Thus the compounds of the invention are useful for immunomodulatory treatment in man, mammals and animals. Included are also 25 conditions associated with increased production of nitric oxide (NO) as well as upregulated activity of nitric oxide synthase (NOS). Moreover, the compounds of the invention are useful for treating conditions related to the testis and ovary.

30 In the present specification "increased production" refers to increased formation, increased release, or increased content of an endogenous compound locally, regionally or systemically in a patient compared to the content of said endogenous compound in a healthy individual. In the present specification "upregulated" refers to an increased activity or content of the compound compared with that in a healthy individual.

35 In the present specification "decreased production" is refers to decreased formation, decreased release, or decreased content of an endogenous compound in a patient

compared to the content of said endogenous compound in a healthy individual. In the present specification "downregulated" refers to a decreased activity or content of the compound compared with that in healthy individual.

- 5 In particular, positive treatment effects or preventive effects are seen in conditions where inflammation or inflammatory like condition is caused by or being associated with one or more of the following: allergy, hypersensitivity, bacterial infection, viral infection, inflammation caused by toxic agent, fever, autoimmune disease, radiation damage by any source including UV-radiation, X-ray radiation,  $\gamma$ -radiation,  $\alpha$ - or  $\beta$ -particles, sun burns,
- 10 elevated temperature, mechanical injury. Moreover, inflammation due to hypoxia, which is optionally followed by reoxygenation of the hypoxic area, is typically followed by severe inflammation, which condition is positively affected by treatment with a compound of the invention.
- 15 In very specific embodiments of the invention a compound of the invention is administered for prevention or therapeutic treatment of inflammatory diseases of the skin (including the dermis and epidermis) of any origin, including skin diseases having a inflammatory component. Specific examples of this embodiment of the invention include treatment of contact dermatitis of the skin, sunburns of the skin, burns of any cause, and inflammation of
- 20 the skin caused by chemical agent, psoriasis, vasculitis, pyoderma gangrenosum, discoid lupus erythematosus, eczema, pustulosis palmo-plantaris, and pemphigus vulgaris.
- Also comprised by the invention is the administration of the compound of the invention for treatment of an inflammatory disease in the abdomen, including an abdominal disease
- 25 having a inflammatory component. Specific examples of treatment of such disease with a compound of the invention are gastritis, including one of unknown origin, gastritis perniciousa (atrophic gastritis), ulcerous colitis (colitis ulcerosa), morbus Crohn, systemic sclerosis, ulcus duodeni, coeliac disease, oesophagitis and ulcus ventriculi.
- 30 Comprised by the invention is also administration of the compound of the invention for treatment of systemic or general and/or local immunological diseases, including those of an autoimmune nature, and other inflammatory diseases of a general nature. Specific examples include treatment of rheumatoid arthritis, psoriatic arthritis, systemic sclerosis, polymyalgia rheumatica, Wegener's granulomatosis, sarcoidosis, eosinophilic fasciitis,
- 35 reactive arthritis, Bechterew's disease, systemic lupus erythematosus, arteritis temporalis, Behcet's disease, morbus Burger, Good Pastures' syndrome, eosinophilic granuloma,

fibromyalgia, myositis, and mixed connective tissue disease. Included therein is also arthritis, including arthritis of unknown origin.

Further included in the invention is administration of the compound of the invention for  
5 treatment of a disease of the peripheral and central nervous system related to  
inflammation. Included in this aspect of the invention is the treatment of cerebral vasculitis,  
multiple sclerosis, autoimmune ophtalmitis, polyneuropathia. Comprised by the invention is  
also the administration of a compound of the invention for treatment of an inflammation of  
10 the central nervous system to prevent apoptotic cell death. Moreover, as the compounds of  
the invention show a distinct ability to induce nerve regeneration, positive treatment effects  
are often seen in central nervous system diseases involving damage of cells in this region.  
This aspect of the invention also includes treatment of traumatic injuries to the central  
nervous system, brain edema, multiple sclerosis, Alzheimer's disease, bacterial and viral  
15 infections in the central nervous system, stroke, and haemorrhagia in the central nervous  
system.

Comprised by the invention is also the administration of the compound of the invention for  
treatment of diseases of the eye and tear glands related to inflammation. Specific examples  
of such diseases comprise anterior and posterior uveitis, retinal vasculitis, optic neuritis,  
20 Wegener's granulomatosis, Sjögren's syndrome, episcleritis, scleritis, sarcoidosis affecting  
the eye and polychondritis affecting the eye.

Comprised by the invention is also the administration of the compound of the invention for  
treatment of diseases of the ear related to inflammation, specific examples of which include  
25 polychondritis affecting the ear and external otitis.

Comprised by the invention is also the administration of the compound of the invention for  
treatment of diseases of the nose related to inflammation, specific examples of which are  
sarcoidosis, polychondritis and mid-line granuloma of the nose.

30 Comprised by the invention is also the administration of the compound of the invention for  
treatment of diseases related to inflammation of the mouth, pharynx and salivary glands.  
Specific examples include Wegener's granulomatosis, mid-line granuloma, Sjögren's  
syndrome and polychondritis in these areas.

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Included in the invention is also the administration of the compound of the invention for treatment of diseases related to inflammation in the lung. Specific examples include treatment of idiopathic alveolitis, primary pulmonary hypertension, bronchitis, chronic bronchitis, sarcoidosis, alveolitis in inflammatory systemic disease, pulmonary hypertension in inflammatory systemic disease, Wegener's granulomatosis and Good Pastures' syndrome.

Comprised by the invention is also the administration of the compound of the invention for treatment of diseases related to the inflammation of the heart. Specific examples include treatment of pericarditis, idiopathic pericarditis, myocarditis, Takayasu's arteritis, Kawasaki's disease, coronary artery vasculitis, pericarditis in inflammatory systemic disease, myocarditis in inflammatory systemic disease, endocarditis and endocarditis in inflammatory systemic disease.

Comprised by the invention is also the administration of the compound of the invention for treatment of diseases related to inflammation of the liver. Specific examples include treatment of hepatitis, chronic active hepatitis, biliary cirrhosis, hepatic damage by toxic agent, interferon induced hepatitis, hepatitis induced by viral infection, liver damage induced by anoxia, liver damage caused by mechanical trauma.

Comprised by the invention is also the administration of the compound of the invention for treatment of diseases related to inflammation of the pancreas. Specific examples include treatment (and prevention) of diabetes mellitus, acute pancreatitis, chronic pancreatitis.

Comprised by the invention is also the administration of the compound of the invention for treatment of diseases related to the inflammation of the thyroid. Specific examples of these embodiments of the invention include treatment of thyroiditis, autoimmune thyroiditis and Hashimoto's thyroiditis.

Comprised by the invention is also the administration of the compound of the invention for treatment of diseases related to inflammation of the kidney. Specific examples include treatment of glomerulonephritis, glomerulonephritis in systemic lupus erythematosus, periarteritis nodosa, Wegener's granulomatosis, Good-Pastures' syndrome, HLAB27 associated diseases, IgA nephritis (IgA = Immunoglobuline A), pyelonephritis, chronic pyelonephritis and interstitial nephritis.

Comprised by the invention is also the administration of the compound of the invention for treatment of diseases related to the inflammation of the joints. Specific examples include treatment of Bechterew's disease, psoriatic arthritis, rheumatoid arthritis, arthritis in colitis ulcerosa, arthritis in morbus Crohn, affection of joints in systemic lupus erythematosus, systemic sclerosis, mixed connective tissue disease, reactive arthritis, Reiter's syndrome. Moreover, included in this embodiment of the invention is treatment of arthrosis of any joint, in particular arthrosis of finger joints, the knee and the hip.

Comprised by the invention is also the administration of the compound of the invention for treatment of diseases related to the inflammation of blood vessels. Specific examples include treatment of arteritis temporalis, periarteritis nodosa, arteriosclerosis, Takayasu's arteritis and Kawasaki's disease. In particular advantageous is the capacity of a compound of the invention to afford protection against and prevention of arteriosclerosis. This is in part due to the capacity of the compound of the invention to prevent the induction of inducible nitric oxide synthase (iNOS) caused by the action of oxidized Low Density Lipoprotein on endothelial cells and blood vessel walls.

Comprised by the invention is also the administration of a compound of the invention for treatment of drug induced disorders of the blood and lymphoid system, including the treatment of drug induced hypersensitivity (including drug hypersensitivity) affecting blood cells and blood cell forming organs (e.g. bone marrow and lymphoid tissue). Specific embodiments of this aspect of the invention include the treatment of anemia, granulocytopenia, trombocytopenia, leukopenia, aplastic anemia, autoimmune hemolytic anemia, autoimmune thrombocytopenia and autoimmune granulocytopenia.

The compounds of the invention can also be administered for treatment of fast allergic disorders (Type I allergy). Included in this embodiment of the invention is the treatment of anaphylactic reactions, anaphylactoid reactions, asthma, asthma of allergic type, asthma of unknown origin, rhinitis, hay fever and pollen allergy.

Comprised by the invention is also the administration of the compound of the invention for the treatment of inflammation related to infections of any origin. Specific examples include treatment of inflammation secondary to infection caused by virus, bacteria, helminths and protozoae.

Comprised by the invention is also the administration of the compound of the invention for treatment of inflammations related to trauma and tissue injury of any origin.

Because of the capacity of the compounds of the invention to stimulate pigment formation in epidermal cells the compounds of the invention are also useful for inducing skin tanning for cosmetic reasons, for treatment of vitiligo, or any other condition where darkening of skin color is desired. Moreover, because of the ability of the compounds of the invention to inhibit pigment formation in cells of the skin they are also useful for inducing lighter skin color for cosmetic reasons, or during any condition where a lighter color of skin is desired.

The compounds of the invention are also useful for inducing formation of the second messenger element cAMP. In particular, such formation of cyclic adenosine 3',5'-monophosphate (cAMP) is desired for eliciting the specific pharmacological effects of the compounds of the invention when administered to a living organism, in particular a human. However, the induction of cAMP formation may also be of great value in cells or crushed cell systems *in vitro*, e.g. for analytical or diagnostic purposes. A specific embodiment of this aspect of the invention is given in Example 3.

The compounds of the invention are also useful for inhibiting the *in vivo* formation of the second messenger element cAMP. Such inhibition may also be used in cells or crushed cell systems *in vitro*, e.g. for analytical or diagnostic purposes.

The compounds of the invention are administered in pharmacologically effective amounts which may vary from 0.001 mg/day/kg body weight to 1000 mg/day/kg body weight depending on the nature of the compound, the desired treatment effect and route of administration; however, lower amounts may be effective, if delivered locally. The compounds of the invention have low toxicity and are well tolerated.

For analytical and diagnostic purposes the compounds of the invention can be used in radioactive form, including radioactive labels. In particular they may be manufactured so as to incorporate radioactive iodine or tritium, or any other suitable radionuclide. Such a radioactively labeled compound can be used in radio-ligand binding for the quantification of MC1-receptors, for the analysis of dissociation constant ( $K_i$ s or  $K_d$ s) of drugs competing with specific subtypes of melanocortin receptors, and for the localization of MC1-receptors in tissues and tissue sections e.g. by use of receptor autoradiographic techniques. Principles of radio-ligand binding and receptor autoradiography are well known in the art.



Alternatively the compounds of the invention can be labeled with any other type of label that allows detection of the respective compound, e.g. a fluorescent label, biotin, an enzyme, and the resulting compound be used for the same purpose as the corresponding radioactively labeled compound. The compound of the invention may also be labeled with  
5 other labels that are activated by secondary processes like irradiation with  $\gamma$ -radiation, light photons or by biochemical processes, the purpose being to cause changes in a target cell or tissue wherein the labeled compound of the invention is located. Such changes may be useful to affect the viability and fate the cells or tissues in an organism to which the compound is administered.

10 The compounds of the invention can also be provided with group that can be activated by light, in particular UV-light, the purpose being to obtain a compound useful for covalent labeling of MC1-receptors by use of the photoaffinity labeling technique. Photoaffinity labeling is a technique well known in the art which in the present context is useful for  
15 elucidating the structure and topological organisation of the MC1- receptor. Such photoactive derivatives of the compounds of the invention are also part of the present invention. Photoactive derivatives of the compounds of the invention may also be made to incorporate and easily detectable group or label, such as e.g. a radioactive atom, a fluorescent group or biotin. (For further details in regard of photoaffinity labeling see Leeb-  
20 Lundberg et al. J. Biol. Hem. 1984, 259, 2579 and Scimonelli & Eberle FEBS Lett. 1978, 226, 134).

The compounds of the invention can also be labeled with gamma or positron emitting isotopes. A thus labeled compound may be administered systematically or locally to an  
25 animal, including a human. By virtue of the ability of the radioactive compound of the invention to bind selectively to MC1- receptors imaging of the *in vivo* binding of specific MC-receptor subtypes may be obtained by using techniques well known in the art such as scintigraphy, positron emission tomography (PET) or single photon emission computed tomography (SPECT). By using such methods information on the distribution and/or  
30 numbers of MC1-receptors in tissues of an animal or a human subject can be obtained. This information is of value for diagnosis of disease, in particular for diagnosis of malignant melanoma, as melanoma cells are known to be rich in MC1-receptors. Moreover, the very same technique may be used for the treatment of any other malignancy where the malignant cells contain MC1-receptors. Examples of the latter are cancer with originating  
35 from the ovaries or testes. For general examples of this technique, which is directly applicable for the compound of the invention, see Bagutt et al. 1994.

Moreover, the compound of the invention can also be tagged with a toxic agent and used for targeted delivery of the toxin to malignant melanoma cells or other cells bearing MC1-receptors (e.g. malignant cells with origin from the ovary or testis). In this context 'tagged' is intended to mean that the compound of the invention is covalently or non-covalently bound to a toxin, for instance ricin, doxorubicin and diphtheria toxin. By virtue of the high affinity and selectivity of the compound of the invention for the MC1-receptor specific delivery of the toxin to MC1-receptor bearing cells (e.g. melanoma cells) is achieved and malignant cell growth is selectively inhibited, even including killing of the malignant cells. The principles used for this approach are well known in the art (Murphy et al. 1986, Tatro et al. 1992, O'Hare et al. 1993). The compounds of the invention may also be tagged with a compound capable of activating the endogenous immune system. A thus formed hybrid of a compound of the invention and a immune system activating compound can be used for treatment of malignant melanoma or any other MC1-receptor expressing disease condition. The portion of the hybrid molecule containing the compound of the invention will, at the one hand, bind to the MC1-receptor of malignant melanoma cells (including both the primary tumor and metastases), as well as other MC1-receptor bearing malignant tumor cells, while, at the other hand, the immune system activating component will trigger an immune response directed against the tumor cells. This turn leads to inhibition of tumor cell growth and eventual tumor cell death. Typical example of such a "bispecific" hybrid compound is a hybrid between the compound of the invention [i.e. the compound according to formula (1), (2), (3) or (4)] and a compound capable of binding to a T-cell antigen. Typically the T-cell antigen binding compound of the hybrid compound is an antibody directed against T-cell antigen CD3. The antibody in this case is preferably a monoclonal antibody. The so formed hybrid compound will direct cytotoxic cells to the malignant melanoma cells or the MC1-receptor bearing malignant cells and inhibit the tumor growth. The compound of the invention may be attached to the antibody chemically by covalent or non-covalent bond(s). The hybrid compound may also be manufactured by genetic engineering by incorporating the DNA sequence encoding a peptide corresponding to the compound of the invention with other DNA encoding a suitable protein. The peptide sequence of the compound of the invention may thus become incorporated into a longer peptide sequence constituting, for instance, an antibody, a hybrid antibody, a hybrid molecule containing part of an antibody, or an antibody like protein. It is furthermore recognized that the hybrid compound may contain one or several copies of the compound of the invention. When several copies of the MC1-receptor binding moieties are incorporated into the hybrid compound of the invention the thus formed hybrid will show increased affinity to the MC1-receptor. This is an

advantage in the treatment of MC1-receptor related malignant disease. In line with what was described above the present invention therefore includes a hybrid compound incorporating one or several copies of the compound of the invention with another molecule. For the general principles underlying immunotherapy using the approach described above reference see Riedle et al. 1998, Mukherji et al. 1995, Canevari et al. 1995 and Thielemans 1995. These approaches and methods can be adopted for targeting MC1-receptor expressing malignant conditions, by use of the approach outlined above.

The compounds of the invention may be used for the treatment and diagnosis of diseases, disorders and/or pathological conditions in an animal, in particular in man.

The compounds of the invention may be delivered to the preferred site in the body by a suitable drug delivery system. For example a compound of the invention may be coupled to a carrier molecule to make it lipophilic (see e.g. Toth, I: Drug Targeting, 1994, 2, 217-239; Patel et al., Bioconjugate Chem 1997, 8, 434-411). Other techniques useful for delivering the compound of the invention to the desired site in the body are vector mediated carrier systems (see e.g. Pardridge, WM: Pharmacol Toxicol 1992, 71, 3-10; Saito, Y et al.: Proc. Natl. Acad. Sci USA 1995, 92, 10227-10231; Wu, D and Pardridge, WM: J. Pharmacol. Exp. Ther. 1996, 279, 77-83). Yet other examples of drug delivery technologies useful for the compounds of the invention include the conjugation of the compound of the invention with an active molecule capable of being transported through a biological barrier (see e.g. Zlokovic, B V: Pharmaceutical Research 1995, 12, 1395-1406). A specific example constitutes the coupling of the compound of the invention to fragments of insulin in order to achieve transport across the blood brain barrier (Fukuta, M et al. Pharmaceutical Res. 1994, 11, 1681-1688). Other examples which are principally useful for and adaptable to the compounds of the present invention are to be found in Prokai-Tatrai, K et al.: J. Med. Chem. 1996, 39, 4775-4782 and Tamai, I et al: J. Pharmacol. Exp. Ther. 1997, 280, 410-415. For general reviews of technologies for drug delivery suitable for the compounds of the invention see Zlokovic, B V: Pharmaceutical Res. 1995, 12, 1395-1406 and Pardridge, W M: Pharmacol. Toxicol. 1992, 71, 3-10.

The present invention also relates to a pro-drug which, upon administration to an animal or a human, is converted to a compound of the invention. A pro-drug of the compound of the invention can be used for the same purposes as described in this patent for the compounds of the invention, as well as is disclosed in the Examples given below.

The compound of the invention can be administered together with peptidase and/or protease inhibitors to prevent or delay the breakdown of the compound of the invention and thereby prolong its duration of pharmacological action in the body as well as its stability in the gastrointestinal tract when administered perorally. Peptidase/protease inhibitors that may be administered together with a compound of the invention are preferably selected from the group of angiotensin converting enzyme inhibitors (ACE-inhibitors) such as e.g. captopril (D-3-mercaptopomethyl-propionyl-L-proline), enalapril, phosphoramidone, amastatin.

- 10 The compounds of the present invention can be bound covalently or non-covalently to one or several of other molecule(s) of any desired structure(s); the thus formed modified compound or complex can be used for the same purposes as described in this patent for the compounds of the invention, as well as is disclosed in the Examples given below. In a particularly important embodiment of the invention a radioactively labeled molecule is
- 15 covalently bound to the compound of the invention so as to make the compound of the invention radioactively labeled.

In the following the invention will be described in greater detail by reference to a number of preferred embodiments which however are only given for purposes of illustration and must not be considered to limit the invention in any way.

### Brief Description of the Figures

#### ~~Legends to Figures~~

- 25 Figure 1: Competition curves for MS05 and MS09 on MC1 and MC3 receptors.

Figure 2: Inhibition of the binding of [<sup>125</sup>I]-NDP-MSH to B16 melanoma cells by MS05 and MS09.

- 30 Figure 3: Stimulation of cAMP-formation in mouse B16 melanoma cells by  $\alpha$ -MSH and MS05.

Figure 4: Inhibition of the binding of [<sup>125</sup>I]-NDP-MSH to mouse RAW 264.7 cells by MS05.

Figure 5: Stimulation of cAMP-formation in mouse RAW 264.7 cells by  $\alpha$ -MSH and MS05.

Figure 6: Inhibition of nitric oxide production in mouse RAW 264.7 cells by  $\alpha$ -MSH, MS05  
5 and MS09.

### Abbreviations

10 Abbreviations used in the following examples:

Fmoc = 9-fluorenylmethoxycarbonyl

DMF = N,N-Dimethylformamide

15

Fmoc-Val-OPfp = 9-Fluorenylmethoxycarbonyl-L-valine pentafluoro phenyl ester

HOAt = 1-Hydroxy-7-azabenzotriazole

20 Fmoc-Pro-OPfp = 9-Fluorenylmethoxycarbonyl-L-proline pentafluorophenyl ester

Fmoc-Lys(Boc)-OPfp =  $\alpha$ -9-Fluorenylmethoxycarbonyl- $\epsilon$ -tert-butyloxycarbonyl-L-lysine  
pentafluorophenyl ester

25 Fmoc-Gly-OPfp = 9-Fluorenylmethoxycarbonyl-glycine pentafluorophenyl ester

Fmoc-Trp(Boc)-OH = 9-Fluorenylmethoxycarbonyl-(N<sup>in</sup>-tert-butyloxycarbonyl)-L-tryptophan

30 HATU = O-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

DIEA = N,N-diisopropylethylamine

35 Fmoc-Arg(Pbf)-OH = 9-Fluorenylmethoxycarbonyl-(N<sup>9</sup>-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-L-arginine

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Fmoc-Phe-OPfp = 9-Fluorenylmethoxycarbonyl-L-phenylalanine pentafluorophenyl ester

Fmoc-His(Trt)-OH = 9-Fluorenylmethoxycarbonyl-N<sup>trityl</sup>-L-histidine

Fmoc-Ser(But)-OH = 9-Fluorenylmethoxycarbonyl-O-tert-butyl-L-serine

Fmoc-Ile-OPfp = 9-Fluorenylmethoxycarbonyl-L-isoleucine pentafluorophenyl ester

10 Ac = acetyl

dPhe = D-phenylalanine

dSer = D-serine

15

NMeSer = N-methyl-L-serine

NMeVal = N-methyl-L-valine

20 NMedPhe = N-methyl-D-phenylalanine

PyAOP = O-(7-azabenzotriazol-1-yl)-tris(pyrrolidino)-phosphonium hexafluorophosphate

25 TFFH = tetramethylfluoroformamidinium hexafluorophosphate

NDP-MSH = [Nle<sup>4</sup>,D-Phe<sup>7</sup>] α-MSH

30 Example 1:1

*Synthesis of L-seryl-L-seryl-L-isoleucyl-L-isoleucyl-L-seryl-L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophanyl-glycyl-L-lysyl-L-prolyl-L-valinamide (MS05) (SEQ ID NO:1):*

The MS05 peptide (Ser-Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH<sub>2</sub>) (SEQ ID NO:1) was assembled on a solid support using the Pioneer peptide synthesis system from PerSeptive Biosystems UK.

- 5 The starting cycle was as follows: 100 mg (0.02 mmole) of [5-(4-Fmoc-aminomethyl-3,5-dimethoxy) valeric acid attached to polyethylene-graft polystyrene support (Fmoc-PAL-PEG-PS, capacity 0.2 mmole/g) was placed into a peptide synthesis column. The Fmoc group was then removed by 5 min treatment with 20 % piperidine in DMF, followed by a wash of the support with DMF.

10

After completion of the starting cycle the resin was subjected to repeated aminoacid coupling cycles, each cycle consisting of 30 - 90 min circulation of appropriate reagents (as detailed below) dissolved in 4 ml DMF through the column, followed by washing with DMF, 5 min treatment with 0.3 M acetic anhydride in DMF, washing

- 15 with DMF, 5 min treatment with 20 % piperidine in DMF, and then again washing with DMF before the start of the next cycle. Thirteen cycles were applied using reagents and treatment times as follows (in order): 1) Fmoc-Val-OPfp (40 mg, 0.08 mmol) and HOAt (11 mg, 0.08 mmol) (90 min), 2) Fmoc-Pro-OPfp (40 mg, 0.08 mmol) and HOAt (11 mg, 0.08 mmol) (60 min), 3) Fmoc-Lys(Boc)-OPfp (51 mg, 0.08 mmol) and HOAt (11 mg, 0.08 mmol) (30 min), 4) Fmoc-Gly-OPfp (37 mg, 0.08 mmol) and HOAt (11 mg, 0.08 mmol) (30 min), 5) Fmoc-Trp(Boc)-OH (42 mg, 0.08 mmol), HATU (30 mg, 0.08 mmol) and DIEA (0.068 ml, 0.4 mmol) (30 min), 6) Fmoc-Arg(Pbf)-OH (52 mg, 0.08 mmol), HATU (30 mg, 0.08 mmol) and DIEA (0.068 ml, 0.4 mmol) (90 min), 7) Fmoc-Phe-OPfp (44 mg, 0.08 mmol) and HOAt (11 mg, 0.08 mmol) (60 min), 8) Fmoc-His(Trt)-OH (50 mg, 0.08 mmol), HATU (30.0 mg, 0.08 mmol) and DIEA (0.068 ml, 0.4 mmol) (30 min), 9) Fmoc-Ser(tBu)-OH (31mg, 0.08 mmol), HATU (30.0 mg, 0.08 mmol) and DIEA (0.068 ml, 0.4 mmol) (30 min), 10) Fmoc-Ile-OPfp (42 mg, 0.08 mmol) and HOAt (11 mg, 0.08 mmol) (90 min), 11) Fmoc-Ile-OPfp (42 mg, 0.08 mmol) and HOAt (11 mg, 0.08 mmol) (90 min), 12) Fmoc-Ser(tBu)-OH (31mg, 0.08 mmol), HATU (30.0 mg, 0.08 mmol) and DIEA (0.068 ml, 0.4 mmol) (60 min), 13) Fmoc-Ser(tBu)-OH (31mg, 0.08 mmol), HATU (30.0 mg, 0.08 mmol) and DIEA (0.068 ml, 0.4 mmol) (30 min).

- 35 After the last cycle the support was washed with DMF, followed by a methanol and methylene chloride wash, and dried in vacuo. The dried resin was treated with 2.5 ml of deprotection mixture (trifluoroacetic acid - phenol - anisole - 1,2-

ethanedithiol - water, 82:2:2:2:2) for 3 hours at room temperature. It was filtered, washed on the filter with trifluoroacetic acid, the filtrates combined and concentrated in vacuo at room temperature. Dry ether was added and the precipitate formed was filtered off and washed on the filter with ether, then dried in vacuo over KOH. Yield 31 mg. HPLC data (2 x 250 mm column, Vydac RP C18, 90A, 201HS1010): k'(main substance) = 3.50 (19% acetonitrile in water + 0.1 % trifluoroacetic acid, detection at 220 nm). The raw product was dissolved in 1 ml of 60 % MeCN in water and the solution divided into three portions and placed into centrifuge tubes, each of them then being diluted with 0.1 % aqueous trifluoroacetic acid to 1.5 ml volume. It was centrifuged and the clear solutions were used for semipreparative HPLC (10 x 250 mm column, Vydac RP C18, 90A, 201HS1010, eluate - 19% acetonitrile in water + 0.1 % trifluoroacetic acid, detection at 220 nm. Fractions containing the main peak were pooled and lyophilized. A white powder was formed. Yield of trifluoroacetate salt of MS-05 was 13.2 mg (33%). R<sub>f</sub> 0.28(1-butanol - pyridine - acetic acid - water, 4:1:1:2). Mass spectrometry data: m/e = 1514.3.

#### Example 1:2

Synthesis of Ser-Ser-Ile-Ile-Ser-His-dPhe-Arg-Trp-Gly-Lys-Pro-Val-NH<sub>2</sub> (MS-09) (SEQ ID NO:2) tetratrifluoroacetate was made essentially as described in Example 1. Yield 36%. R<sub>f</sub> 0.56. k' 2.0 (21% MeCN in 0.1% TFA). m/e 1512.9.

#### Example 1:3

Synthesis of Tyr-Ser-Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH<sub>2</sub> (MS-30) (SEQ ID NO:3) tetratrifluoroacetate was made essentially as described in Example 1. Yield 33%. R<sub>f</sub> 0.79. k' 4.4 (8.4% MeCN in 0.1% TFA). m/e 1675.9.

#### Example 1:4

Synthesis of Tyr-Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH<sub>2</sub> (MS-31) (SEQ ID NO:4) tetratrifluoroacetate was made essentially as described in Example 1. Yield 34%. R<sub>f</sub> 0.75. k' 2.7 (10.8% MeCN in 0.1% TFA). m/e 1589.1.



**Example 1:5**

Synthesis of Ser-Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Tyr-NH<sub>2</sub> (MS-32) (SEQ ID NO:5) tetratrifluoroacetate was made essentially as described in Example 1.

5 Yield 28%. R<sub>f</sub> 0.75. k' 3.3 (9.6% MeCN in 0.1% TFA). m/e 1675.9.

**Example 1:6**

Synthesis of Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH<sub>2</sub> (MS-33) (SEQ ID NO:6) tetratrifluoroacetate was made essentially as described in Example 1.

10 Yield. 26%. R<sub>f</sub> 0.73. k' 3.6 (8.4% MeCN in 0.1% TFA). m/e 1425.7.

**Example 1:7**

15 Synthesis of Thr-Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH<sub>2</sub> (MS-34) (SEQ ID NO:7) tetratrifluoroacetate was made essentially as described in Example 1.

Yield 52%. R<sub>f</sub> 0.72. k' 2.4 (10.8% MeCN in 0.1% TFA). m/e 1527.1.

**Example 1:8**

20

Synthesis of Ser-Thr-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH<sub>2</sub> (MS-35) (SEQ ID NO:8) tetratrifluoroacetate was made essentially as described in Example 1.

Yield 49%. R<sub>f</sub> 0.68. k' 2.2 (10.8% MeCN in 0.1% TFA). m/e 1526.8.

25 **Example 1:9**

Synthesis of Ser-Ser-Val-Ile-Ser-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH<sub>2</sub> (MS-36) (SEQ ID NO:9) tetratrifluoroacetate was made essentially as described in Example 1.

Yield 42%. R<sub>f</sub> 0.68. k' 2.3 (10.8% MeCN in 0.1% TFA). m/e 1499.3.

30

**Example 1:10**

Synthesis of Ser-Ser-Ile-Val-Ser-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH<sub>2</sub> (MS-37) (SEQ ID NO:10) tetratrifluoroacetate was made essentially as described in Example 1.

35 Yield 69%. R<sub>f</sub> 0.65. k' 3.3 (12.0% MeCN in 0.1% TFA). m/e 1499.0.

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**Example 1:11**

Synthesis of Ac-Ser-Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH<sub>2</sub> (MS-38) (SEQ ID NO:11) tetratrifluoroacetate was made essentially as described in Example 1.

5 Yield 49%. R<sub>f</sub> 0.72. k' 1.8 (10.8% MeCN in 0.1% TFA). m/e 1554.7.

**Example 1:12**

Synthesis of dSer-Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH<sub>2</sub> (MS-39) (SEQ ID NO:12) tetratrifluoroacetate was made essentially as described in Example 1.

10 Yield 44%. R<sub>f</sub> 0.69. k' 2.2 (12% MeCN in 0.1% TFA). m/e 1512.4.

**Example 1:13**

15 Synthesis of NMeSer-Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH<sub>2</sub> (MS-40) (SEQ ID NO:13) tetratrifluoroacetate was made essentially as described in Example 1.

Fmoc-NMeSer(Bu<sup>t</sup>)-OH was added using PyAOP.

Yield 47%. R<sub>f</sub> 0.67. k' 1.7 (12% MeCN in 0.1% TFA). m/e 1526.6.

**Example 1:14**

Synthesis of Ser-Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Lys-Pro-NMeVal-NH<sub>2</sub> (MS-41) (SEQ ID NO:14) tetratrifluoroacetate was made essentially as described in Example 1.

Fmoc-NMeVal-OH was added using TFFH.

25 Yield 2.5%. R<sub>f</sub> 0.68. k' 4.4 (22.8% MeCN in 0.1% TFA). m/e 1527.3.

**Example 1:15**

30 Synthesis of Ser-Ser-Ile-Ile-Ser-His-NMedPhe-Arg-Trp-Gly-Lys-Pro-Val-NH<sub>2</sub> (MS-42) (SEQ ID NO:15) tetratrifluoroacetate was made essentially as described in Example 1.

Fmoc-NMedPhe-OH was added using PyAOP.

Yield 40%. R<sub>f</sub> 0.70. k' 2.5 (10.8% MeCN in 0.1% TFA). m/e 1527.0.

## Example 2

### *Assay of binding affinities of compounds of the invention for human MC-receptors*

- 5 Expression of receptor clones. Human MC1- and MC5-receptor DNAs (Chhajlani and Wikberg 1992; Chhajlani et al., 1993), cloned into the expression vector pRc/CMV (InVitrogen Corp., USA), and human MC3- and human MC4-receptor DNAs (Gantz et al., 1993a & b), cloned into the expression vector pCMV/neo, were used. COS cells were grown and transfected with receptor clones as described (Schiöth et al. 1995, 1996b).
- 10 After transfection cells were cultivated for 48 h, detached from the Petri dishes, and used for radioligand binding as described (Schiöth et al. 1995, 1996b).

- Binding studies. The transfected cells were washed with binding buffer (Minimum Essential Medium with Earle's salts, 25 mM HEPES, pH 7.0, 0.2 % bovine serum albumin and
- 15 distributed into 96 well plates. The cells were then incubated for 2 h at 37°C, with 0.1 ml binding buffer in each well containing [<sup>125</sup>I][Nle<sup>4</sup>, D-Phe<sup>7</sup>]α-MSH and appropriate concentrations of the peptide to be tested. After incubation the plates were put on ice and the cells were washed with 0.1 ml of ice-cold binding buffer. The cells were then detached from the plates with 0.2 ml of 0.1 N NaOH. Radioactivity was counted by using a Wallac,
- 20 Wizard automatic gamma counter. The competition data were analyzed by fitting it to the logistic function using non-linear regression analysis. The K<sub>i</sub>-values were then calculated from the thus obtained IC<sub>50</sub>-values by using the Cheng and Prusoff equation, essentially as described by Schiöth et al. 1995, 1996b.

## 25 Results

K<sub>i</sub> values for NDP-MSH, α-MSH, MS05 and MS09 for MC1, MC3, MC4 and MC5 receptors are shown in the Table below:

30

35

<u>Compound</u>	<u>MC1</u>	<u>MC3</u>	<u>MC4</u>	<u>MC5</u>
	$K_i$ (nM)	$K_i$ (nM)	$K_i$ (nM)	$K_i$ (nM)
NDP-MSH	0.11	0.39	2.3	2.9
$\alpha$ -MSH	0.68	52.3	2030	4990
MS05	0.76	1365	>>50000	>>50000
MS09	0.20	7.0	40	120

Figure 1 shows competition curves for MS05 and MS09 on MC1 and MC3 receptors.

### Example 3

*Assays of the effect of the compounds of the invention on generation of the second messenger cAMP in cells expressing MC1-receptors*

#### Preparation of stable cell lines

Human MC1-receptors had been cloned earlier into pZeoSV (Invitrogen) (Szardenings *et al.*, 1997). The resulting vectors were used for transformation of the amelanotic mouse melanoma cell line B16G4F that lacks the mouse MC1-receptors (Solca *et al.*, 1993) using liposomes (Campbell, 1995) as described previously (Schiöth *et al.*, 1997b). Cells were cultivated in Dulbecco's modified Eagle's medium with 10 % fetal calf serum at 7% CO<sub>2</sub> for 24 hours and seeded on large plates in the same medium containing 300 µg/ml Zeocin. The medium was exchanged every 4-5 days until cell foci appeared. Single foci were isolated and grown under antibiotic selection. Expression and binding characteristics of these receptors were determined and found to be identical with the same receptors expressed in other cell lines, as has already also been shown earlier for the human MC1-receptor (Chluba-de-Tapia *et al.* 1996).

#### Stimulation of cell cAMP

For cAMP measurements the cells were detached from 60-80% confluent adherent cultures using Hank's balanced salts containing 0.5 mM EDTA and incubated for 30-60 min at 37°C in ordinary growth medium containing 0.5 mM of the phosphodiesterase inhibitor

3-*iso*-butyl-1-methyl-xanthine (IBMX). 20 µl aliquots of appropriate dilutions of test compounds in growth medium were prepared in 96 well microtitre plates and placed in a water bath at 37°C. For the stimulation about  $1.5 \times 10^5$  cells in 180 µl were quickly added to each well to obtain immediate mixing. After 20 min 20 µl of 4.4 M perchloric acid were added, mixed, neutralized after a few minutes by addition of 20 µl base (5 M KOH, 1 M Tris) and centrifuged.

#### Determination of cAMP concentrations

20 µl of acid treated supernatant obtained above were mixed with 50 µl buffer (100 mM Tris-Cl, 250 mM NaCl, 10 mM EDTA, 0.1% mercaptoethanol, 0.5 mM IBMX, pH=7.4) containing 0.01 µCi [ $^3\text{H}$ ]cAMP (Amersham, 1.04 TBq/mmol, 1 µCi/µl, product no.: TRK304). 200 µl of the same buffer containing a 1:16 diluted porcine adrenal gland bark extract (prepared as described by Nordstedt and Fredholm, 1990) were added and the microtitre plates were incubated for at least 2 hours at 4°C. A standard curve was prepared in the same manner with dilutions of cAMP covering the range 2 µM - 0.5 nM.

After completion of incubation the solutions were filtered over GF-B glassfibre filters (Whatman) and washed briefly with ca. 2 ml ice-cold washing buffer (50 mM Tris-Cl, pH=7.4). Radioactivity on the filters was measured after addition of scintillation liquid. Stimulation experiments were determined in quadruplicates and standard curves in duplicates.

#### Results of tests of MS05 and MS09 on cAMP formation in the MC1-receptor expressing cells

The magnitude of stimulation of cAMP formation in the treated MC1-receptor expressing cells compared to the cAMP content in untreated control cells were tested for 10 nM and 1 µM MS05 and MS09, and were as given in the below table. For comparison the stimulation obtained by NDP-MSH, α-MSH, forskolin and MS04 (Szardenings et al. 1997 ) is also given.

	<u>Compound</u>		<u>Fold stimulation</u>	<u>% of 10 nM</u>
			<u>of cAMP</u>	<u><math>\alpha</math>-MSH</u>
5	NPD-MSH	(10 nM)	$7.4 \pm 0.7$	123 %
	NPD-MSH	(1 $\mu$ M)	$5.9 \pm 0.4$	
	$\alpha$ -MSH	(10 nM)	$6.2 \pm 0.7$	100 %
	$\alpha$ -MSH	(1 $\mu$ M)	$5.0 \pm 0.6$	
	MS04	(10 nM)	$1.0 \pm 0.1$	0 %
10	MS04	(1 $\mu$ M)	$4.6 \pm 0.4$	
	MS05	(10 nM)	$4.6 \pm 0.3$	69 %
	MS05	(1 $\mu$ M)	$6.7 \pm 0.3$	
	MS09	(10 nM)	$5.5 \pm 0.9$	89%
	MS09	(1 $\mu$ M)	$7.0 \pm 0.4$	
15	Forskolin	(1 $\mu$ M)	$7.3 \pm 1.1$	
	Control		1	

In the above table the foldness of stimulation (compared to untreated control cells) is shown as the mean  $\pm$  standard deviation of 3 independent experiments. Shown is also the ability of 10 nM of a compound to stimulate cAMP calculated in percent (%) of the capacity of 10 nM of  $\alpha$ -MSH to induce stimulation of cAMP. It is evident from the above table that both MS05 and MS09 are effective stimulators of cAMP formation at both 10 nM and 1  $\mu$ M concentration. Note that the MS04 peptide is completely devoid of effect on cAMP at 10 nM and 1  $\mu$ M of MS04 is required to induce stimulation of cAMP formation. Both MS05 and MS09 are highly effective stimulators of cAMP at 10nM.

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#### Example 4

*Demonstration of the capacity of the compounds of the invention to bind to melanocortin (MSH) receptors in mouse B16 melanoma cells*

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#### Cell culture

B16 mouse melanoma cells (B16-F1; CR-6323, American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, 20852) were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Gaithersburg, USA, cat no. 041-01966H) supplemented with 10% heat inactivated fetal bovine serum, 100 IU penicillin/ml and 100 microgram streptomycin/ml at

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37°C in a humidified atmosphere of 95 % air and 5 % CO<sub>2</sub>. Cells grown in monolayers were detached from the culture flasks using Hank's balanced salts containing 0.5 mM EDTA and collected by low speed centrifugation (700 x g).

## 5 Receptor binding studies

Assays for MSH-receptor binding was done essentially as described (Xia et al., Cancer Letters, 1996, 98, 157-162), in principle according to earlier described methods (Eberle et al., J. Recept. Res. 1991, 11, 311-322). In brief, collected cells were washed with binding buffer (for composition of binding buffer see below) distributed into 96 well plates and sedimented onto the well bottoms by centrifugation. The cells were then incubated for 2 h at 37°C, with 0.1 ml binding buffer in each well, the buffer containing [<sup>125</sup>I][Nle<sup>4</sup>, D-Phe<sup>7</sup>] α-MSH (0.2nM), different concentrations of the test compound in different wells at 37°C in binding buffer of the following composition MEM (MEM = Minimum Essential Medium, Gibco BRL, Gaithersburg, USA, cat no.041-01095H) with Eagle's salts, 25 mM HEPES, pH 7.4, 0.2 % bovine serum albumin, 1 mM 1,10-phenanthroline, 0.5 microgram leupeptin/ml and 200 microgram bacitracin/ml. After incubation the plates were put on ice, centrifuged and the cells washed with 0.1 ml of ice-cold binding buffer, centrifuged and the binding buffer was sucked off. The finally sedimented and washed cells were then detached from the plates with 0.2 ml of 0.1 N NaOH. Radioactivity was counted by using a Wallac, Wizard automatic gamma counter. The competition data were analysed by law of massaction computer modelling essentially as described (Bergstrom & Wikberg, Acta Pharmacol. Toxicol. 1986, 59, 270-278).

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## Results

As is seen from Fig. 2 both the MSO5 and the MSO9 peptide caused a dose dependent inhibition of the binding of [<sup>125</sup>I]-NDP-MSH to the B16 melanoma cells. Analysis of data by law of massaction computer modelling indicated that the MSO5 and MSO9 peptides competed with [<sup>125</sup>I]-NDP-MSH at one single class of binding site (i.e. the B16 melanoma cell MSH-receptor, in other words the native mouse MC1-receptor). The K<sub>i</sub> of MSO5 for the mouse B16 melanoma cell MSH-receptor was estimated to be 1.00 ± 0.11 nM (mean ± SEM; n=2). The K<sub>i</sub> of MSO9 for the mouse B16 melanoma cell MSH receptor was estimated to be 0.060 ± 0.0035 nM (mean ± SEM; n=2).

### Example 5

*Demonstration of the capacity of the compounds of the invention to afford stimulation of cAMP-formation in mouse B16 melanoma cells*

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#### Cell culture

10 B16 mouse melanoma cells (B16-F1; CRL-6323, American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, 20852), were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Gaithersburg, USA, cat no. 041-01966H) supplemented with 10% heat-inactivated fetal bovine serum, 100 IU penicillin/mL and 100 µg streptomycin/mL at 37°C in a humidified atmosphere of 95 % air and 5 % CO<sub>2</sub>. Cells grown in monolayers were detached from 60-80% confluent adherent cultures using Hank's balanced salts containing 0.5 mM EDTA and collected by low speed centrifugation (700 x g).

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#### Stimulation of cell cAMP

20 The cells were incubated for 30 min at 37°C in DMEM containing 0.5mM of the phosphodiesterase inhibitor 3-iso-butyl-1-methyl-xanthine (IBMX). 20 µL aliquots of appropriate dilutions of test compounds in growth medium were prepared in 96 well microtitre plates. The plates were then placed in a water bath at 37°C and about  $1.5 \times 10^5$  cells in 180 µL were quickly added to each well to obtain immediate mixing. After 20 min, 20 µL 4:4 M perchloric acid was added to each well with mixing, and after a few minutes 20 µL base (5 M KOH, 1 M Tris) was added to each well, whereafter the plates were  
25 centrifuged, and the supernatants collected.

#### Determination of cAMP concentrations

30 Twenty µL of the above supernatants were mixed with 50 µL cAMP assay buffer (100 mM Tris-Cl, 250 mM NaCl, 10 mM EDTA, 0.1% mercaptoethanol, 0.5 mM IBMX, pH 7.4) containing 0.01 µCi [<sup>3</sup>H] cAMP (Amersham, 1.04 TBq/mmol, 1 µCi/µL, product no.: TRK304) in 96-microtiter plate wells. 200 µL of cAMP assay buffer containing a 1:16 diluted porcine adrenal gland bark extract (prepared as described by Nordstedt and Fredholm, Anal. Chem, 1990, 189, 231-234) was then added into each well, and the  
35 microtitre plates were incubated for at least 2 hours at 4°C. A standard curve was prepared



by exchanging the supernatants with cAMP standards covering the concentration range 2  $\mu$ M - 0.5 nM of final cAMP in the assays.

After completion of the incubations the solutions were rapidly filtered onto GF-B glassfibre filters (Whatman), followed by rapid washing of filters with 2 ml ice-cold washing buffer (50 mM Tris-Cl, pH 7.4). Filters were then placed into scintillation vials and a scintillation cocktail was added. Radioactivity was counted using a  $\beta$ -counter. Cell experiments were determined in quadruplicates and standard curves in duplicates.

## 10 Results

The results from the test of  $\alpha$ -MSH and MSO5 peptide on cAMP levels in mouse B16 melanoma cells is shown in Fig. 3. As can be seen from the figure, both peptides caused a marked increase in cAMP at similar potencies and efficacies.

## 15 Example 6

*Demonstration of the capacity of the compounds of the invention to bind to melanocortin (MSH) receptors in RAW 264.7 macrophage cells.*

### Cell culture

RAW 264.7 cells (TIB-71) were obtained from American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, 20852, USA, and cultured in Dulbecco's modified Eagle medium (DMEM; Gibco-BRL, Gaithersburg, USA, cat no. 041-01966H) supplemented with 10% heat-inactivated fetal bovine serum, 100 IU penicillin/ml and 100  $\mu$ g streptomycin/ml at 37°C in a humidified atmosphere of 95 % air and 5 % CO<sub>2</sub>. Cells grown in monolayers were detached from the culture flasks and collected by low speed centrifugation (700 x g).

### 30 Receptor binding studies

MSH-receptor binding was done essentially as described (Xia et al., Cancer Letters, 1996, 98, 157-162), in principle according to earlier described methods (Eberle et al., J. Recept. Res. 1991, 11, 311-322). In brief, the collected cells were washed, distributed into 96 well plates and sedimented onto the well bottoms by centrifugation. The cells were then

incubated for 2 h at 37°C, with 0.1 ml binding buffer in each well containing [<sup>125</sup>I][Nle<sup>4</sup>, D-Phe<sup>7</sup>] α-MSH (0.1 nM), different concentrations of the test compound in different wells at 37°C in MEM (Minimum Essential Medium) with Eagle's salts, 25 mM Hepes, pH 7.4, 0.2% bovine serum albumin, 1 mM 1,10-phenanthroline, 0.5 microgram leupeptin/ml and 200 microgram bacitracin/ml. After incubation the plates were put on ice, centrifuged and the cells washed with 0.1 ml of ice-cold binding buffer, centrifuged and the binding buffer was sucked off. The finally sedimented and washed cells were then detached from the plates with 0.2 ml of 0.1 N NaOH. Radioactivity was counted by using a Wallac, Wizard automatic gamma counter. The competition data were analysed by law of massaction computer modelling essentially as described (Bergstrom & Wikberg, Acta Pharmacol. Toxicol. 1986, 59, 270-278).

### Results

As is seen from Fig. 4, the MSO5 peptide caused a dose dependent inhibition of the binding of [<sup>125</sup>I]-NDP-MSH to the mouse RAW 264.7 cells. Analysis of data by law of massaction computer modelling indicated that MSO5 competed with [<sup>125</sup>I]-NDP-MSH at one single class of binding site (i.e. the RAW 264.7 cell MSH-receptor, which in other words is the native mouse MC1 receptor of the RAW 264.7 cells). The dissociation constant (K<sub>d</sub>) of MSO5 for the binding site was estimated to be 1.35 ± 0.61nM (mean ± SEM).

### Example 7

*Demonstration of the capacity of the compounds of the invention to afford stimulation of cAMP-formation in mouse RAW 264.7 macrophage cells*

RAW 264.7 cells were grown as described in Example 6. cAMP was determined essentially as described in Example 5. Results for α-MSH and MSO5 peptide are shown in Fig. 5. As can be seen from the Figure, both peptides caused a marked increase in cAMP at similar potencies and efficacies.

### Example 8

*Demonstration of the capacity of the compounds of the invention to inhibit nitric oxide  
5 production in mouse RAW 264.7 macrophage cells.*

#### Cell culture

RAW 264.7 cells (TIB-71), obtained from American Type Culture Collection, 12301  
10 Parklawn Drive, Rockville, MD, 20852, USA, and cultured in Dulbecco's modified Eagle  
medium (Gibco, BRL) supplemented with 10% heat-inactivated fetal bovine serum, 100 IU  
penicillin/ml and 100µg streptomycin/ml at 37°C in a humidified atmosphere of 95% air and  
5% CO<sub>2</sub>. Cells grown in monolayers were detached from the culture flasks and collected by  
low speed centrifugation (700 x g).

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#### Incubation of compounds of the invention with RAW 264.7 cells

The cells obtained as above were resuspended in F-12 (HAM) medium (Gibco, BRL) and  
distributed into 96-well plates at a density of  $2.5 \times 10^6$  cells per well, and incubated with 100  
20 ng/mL bacterial lipopolysaccharide (L-4391, Sigma Chemical Company, P.O. Box 14508,  
St. Louis, Missouri 63178, USA), 5 units/mL of mouse recombinant interferon gamma (I-  
5517, Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri 63178, USA) and  
the compounds of the invention using concentrations ranging 0 - 1 µM, for 16 h, whereafter  
an aliquot of the medium was collected for measurement of nitric oxide (NO).

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#### Measurement of nitric oxide

Nitric oxide was measured by monitoring the nitrite production essentially using the method  
of Wishnok et al. (Methods in Enzymology, 1996, 268, 130-151). In brief 50µL of culture  
30 medium was mixed with 50µL Griess reagent (i.e. a 1:1 mixture of 0.1 % N-  
naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% (v/v) phosphoric  
acid) and after 10 min the absorption was measured at 540 nm. The nitrite concentrations  
were calculated from a standard curve constructed, by instead of culture medium, adding  
50µL of between 3 to 100µM of NaNO<sub>2</sub> to the assays.

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### Results

The results are shown in Fig. 6. As can be seen from the Figure, the compounds of the invention, MSO5 and MSO9, as well as  $\alpha$ -MSH caused a strong dose dependent inhibition of the NO-production, the potencies and efficacies of MSO5 and MSO9 being similar to that of the  $\alpha$ -MSH. This data shows that MSO5 and MSO9 share the capacity of  $\alpha$ -MSH to inhibit inflammation. This is because NO is a key component of inflammation.

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